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ABSTRACT

of the dissertation for the degree of Doctor of Philosophy

DIAGNOSIS, GENETIC HETEROGENEITY AND INCIDENCE OF GALACTOSEMIA, INHERITED METABOLIC DISORDER IN NEWBORNS IN BAKU CITY

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INTRODUCTION

The relevance of the topic and the degree of development. Galactosemia is an autosomal recessive inherited disorder accompanied by metabolic disorders from the first days of postnatal development of newborns as a result of enzyme deficiency caused by allelic variants of the *GALT* (9p13.3; 4.3 kb; 11 exons), *GALK1* (17q25.1; 7.3 kb; 8 exons), *GALE* (1p36.11; 5 kb13) and *GALM* (2p22.1; 68 kb; 7 exons) genes[1, 2]. The main source of galactose is the disaccharide lactose, which is present in high amounts in milk and dairy products. Under the influence of the lactase enzyme, it is broken down into glucose and galactose in the intestine, after which galactose is absorbed into the blood and converted to glucose in the liver. In the absence of the galactose-1-phosphate uridylyltransferase enzyme, which is an expression product of the *GALT* gene and plays a key role in the metabolic process, galactose is not converted to glucose, and as a result of the body's failure to absorb galactose sugar, its increased blood level poisons the brain, causing galactosemia oligophrenia in the patient, cataracts in the eyes, hepatomegaly and cirrhosis of the liver, and retardation of physical and mental development[3]. The disorder in the first days of the newborn is accompanied by jaundice, neurological symptoms (convulsions, nystagmus, muscle hypotonia), vomiting, and later, physical and mental developmental delay[4].

It is known that, depending on the nature of the mutation and its

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[1] Kikuchi, A.; Wada, Y.; Ohura, T.; Kure, S. The Discovery of GALM Deficiency (Type IV Galactosemia) and Newborn Screening System for Galactosemia in Japan. *Int. J. Neonatal Screen.* 2021, *7*, 68. [Google Scholar] [CrossRef] [PubMed]

[2] Iwasawa, S.; Kikuchi, A.; Wada, Y.; Arai-Ichinoi, N.; Sakamoto, O.; Tamiya, G.; Kure, S. The prevalence of GALM mutations that cause galactosemia: A database of functionally evaluated variants. *Mol. Genet. Metab.* 2019, *126*, 362–367. [Google Scholar] [CrossRef] [PubMed]

[3] Berry, G.T. Classic Galactosemia and Clinical Variant Galactosemia. In *Gene Reviews*; Adam, M.P., Mirzaa, G.M., Pagon, R.A., Wallace, S.E., Bean, L.J.H., Gripp, K.W., Amemiya, A., Eds.; University of Washington: Seattle, WA, USA, 2000; pp. 1993–2022. Available online: https://www.ncbi.nlm.nih.gov/books (accessed on 20 May 2022).

[4] Bosch A.M. Classical galactosaemia revisited. J. Inherit. Metab. Dis. 2006; 29(4): 516–25.

position in the gene structure (location in the regulatory region and within the exon), translation or folding processes can be disrupted by 10–90%, which causes variability in the clinical course of the disorder and can also lead to the accumulation of pathogenic toxic intermediary products in tissues and vital organs, disrupting the normal development of the body and leading to early death[5]. In addition, in many cases, mutations are neutral and, although accompanied by genetic polymorphism, do not affect the structure of the protein to be formed. In this regard, molecular-genetic screening of mutations in the *GALT* gene that cause classical galactosemia, investigation of their role in changing the activity of galactose-1-phosphate uridylyltransferase enzyme, and study of their impact on the clinical course of the disorder are extremely important for the correct diagnosis, prevention, and determination of therapeutic approaches for this inherited disorder.

The fact that the most common form[6] of galactosemia, caused by mutations in the *GALT* gene, has not been studied in the population of the Republic of Azerbaijan to date, the frequency of occurrence has not been determined, the lack of fundamental information about its genetic nature, and the lack of identification of allelic variants justify the relevance of the topic of the current research work and determine the inevitability of its implementation.

Object and subject of research. The research investigated the inherited metabolic disorder galactosemia in 576 (299 boys and 277 girls) newborns and 138 (70 boys and 68 girls) children with suspected metabolic disorder.

Goals and objectives of the research. The aim of the research was to identify the inherited metabolic disorder — galactosemia — in newborns and children with suspected metabolic disorder in Baku, as well as to conduct a comparative analysis of its genetic nature,

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[5] Rubio-Gozalbo M.E., Haskovic M., Bosch A.M., Burnyte B., Coelho A.I., Cassiman D., Couce M.L., Dawson C., Demirbas D., Derks T. The natural history of classic galactosemia: Lessons from the GalNet registry. Orphanet J. Rare Dis. 2019;14:86. doi: 10.1186/s13023-019-1047-z. *[DOI] [PMC free article] [PubMed] [Google Scholar]*

[6] Berry, G.T. Classic Galactosemia and Clinical Variant Galactosemia. In Gene Reviews; Adam, M.P., Mirzaa, G.M., Pagon, R.A., Wallace, S.E., Bean, L.J.H., Gripp, K.W., Amemiya, A., Eds.; University of Washington: Seattle, WA, USA,

biochemical polymorphism, clinical features, and frequency of occurrence among newborns and patient children. To achieve this goal, the following tasks have been set:

1. To conduct screening for galactosemia - inherited metabolic disorder among newborns and children with suspected metabolic disorder based on the immunoenzyme analysis method;

2. To study biochemical blood parameters associated with galactosemia in newborns and children with suspected metabolic disorder;

3. To identify the molecular structure of the *GALT* gene in detected patients, determine allelic variants and the type of inheritance of the disorder;

4. To compare allelic variants of the *GALT* gene with the activity indicators of the galactose-1-phosphate uridyltransferase enzyme and to determine the relationships between the mutations detected in this way and gene expression and protein polymorphisms;

5. To determine the frequency of occurrence of galactosemia among newborns and patient children;

6. To study the clinic of the disorder in children with galactosemia who suffer from any other inherited metabolic disorder or disorders;

7. To investigate polymorphism of the *MCM6* enhancer of the *LCT* gene, which may cause hypolactosis in patients with suspected galactosemia;

8. To propose preventive measures for galactosemia in newborns.

Methods of the research. In the research, galactosemia was screened using the immunoenzyme analysis method and NGS (Next Generation Sequencing) technology, and statistical analysis of the results was performed.

The main provisions presented for the defense are:

1. The identification of galactosemia, an inherited metabolic disorder, in newborns in Baku confirms its presence in the studied population;

2. The transitions c.563 (A→G), c.974 (C→T) and c.997 (C→T) of the *GALT* gene (respectively, p.188 (Gly→Arg), p.325 (Pro→Leu), p.333 (Arg→Trp) amino acid substitutions) lead to a severe clinical phenotypic manifestation of the disorder by disrupting the native structure of the protein;

3. The transition c.940 (A→G) (p. 314 (Asn→Asp)) of exon 10 of the *GALT* gene causes partial activity of the galactose-1-phosphate uridylyltransferase enzyme in the homozygous inheritance type, which is accompanied by the Duarte of the disorder - a mild clinical form.

4. The heterozygous detection of GTCA deletion at position c.-119_ -116 in the 5' untranslated region (UTR) of the *GALT* gene in two consanguineous married couples, in which the cause of infant death of unknown cause was observed in second-degree family members, suggests that this missense mutation, which causes gene repression, along with the above-mentioned null alleles, is also typical for the Azerbaijani population.

5. Identification of the point mutations of disorder and characterization of its clinical manifestations is important in considering molecular genetic screening during family planning and in predicting the healthy birth of the next child in families with risk groups.

6. The mutations detected allow to offer appropriate therapies, by using as passport data, to newborns and children with suspected metabolic disorders.

Scientific novelty of the research. For the first time, the galactosemia – inherited metabolic disorder - was identified among newborns and children with suspected metabolic disorder in Baku through the complex application of the molecular structure sequence of the *GALT* gene and immunoenzyme analysis methods. The frequency of the disorder among newborns and children with suspected metabolic disorder was studied, and the typical allele profiles and nature of the alleles for the local population were characterized. Among newborns, both the lethal null alleles of the *GALT* gene caused by missense mutations, the phenotypically undetectable neutral allele due to the formation of a synonymous codon, and isoalleles were identified, severe and Duarte clinical forms of the disorder were distinguished, and lethal allele carriers were detected, which confirms the prevalence of genetic carriers of the disease in the Azerbaijani population and the importance of implementing preventive screening programs against the disorder. The detection of mutations in the *GALT*, *IDS* and *ABCC2* genes

demonstrates the possibility of joint observation of multi-genetic pathologies and proves the necessity of applying complex genetic analyses in inherited metabolic disorders.

Theoretical and practical significance of the research. For the first time in Azerbaijan, the carrier and frequency of occurence of galactosemia have been determined as a result of the complex application of immunoenzyme analysis and molecular-genetic screening, which confirms the necessity of developing early screening programs for inherited metabolic disorder of newborns, including galactosemia, and serves to prevent the clinical complications of the disorder based on the therapy to be applied in the postnatal period.

The specific mutation spectrum discovered in the *GALT* gene allows for the design of targeted diagnostic panels and the application of local genetic tests that are more economically efficient for the country's population. The identification of a missense mutation based on an A→G transition (p.188 Gly→Arg) at position 563 of the *GALT* gene in both homozygous and heterozygous cases in newborns and children with suspected metabolic disorder, as well as the detection of a GTCA deletion at position c.-119_ -116 in the 5' UTR in heterozygous cases in each member of two consanguineous pairs, provides grounds to recommend the use of these null alleles as diagnostic markers.

The co-occurrence of multi-genetic pathologies in children with suspected inherited metabolic disorder indicates the importance of applying extensive genetic analyses during differential diagnosis and makes it urgent to expand the scope of genetic counseling in healthcare. In this regard, the introduction of expanded genetic sequencing tests for children suffering from metabolic disorders is considered appropriate.

The obtained data prove the importance of including galactosemia identification in the genetic registry of inherited metabolic disorders and its use in genetic counseling centers.

Preventive measures such as conducting disorder screening in maternity homes and children's hospitals in the republic, organizing educational conversations with parents of patient children, providing medical-genetic counseling to families at genetic risk, and performing

prenatal diagnostics of the fetus in the womb during pregnancy will serve to completely eliminate the risk of having patient children.

The results of the study can be used as initial information for disease screening, determination of familial carrier status and organization of genetic counseling services.

Approbation and application of the work. Main results of the dissertation were presented at the VII International Scientific Conference of Young Scientists and Researchers on "Innovation Problems of Modern Biology" dedicated to the 94th anniversary of the birth of the Great Son of the Azerbaijani People, National Leader Heydar Aliyev (Baku, 2017), at the international scientific conference of young scientists and students on the topic "Innovations in Biology and Agriculture to Solve Global Challenges" dedicated to the 90th anniversary of Academician Jalal Aliyev (Baku, 2018), at the Republican Scientific Conference of Doctoral Students and Young Researchers dedicated to the 100th anniversary of the Azerbaijan Democratic Republic (Baku, 2018), at the VII Republican Scientific Conference on "Actual Problems of Ecology and Soil Science in the 21st Century" dedicated to the 95th anniversary of the birth of the National Leader Heydar Aliyev (Baku, 2018), at the XXIII Republican Scientific Conference of Doctoral Students and Young Researchers dedicated to the 650th anniversary of the great Azerbaijani poet Imadaddin Nasimi, organized at the Azerbaijan University of Architecture and Construction (Baku, 2018), at the international scientific conference held in Latin America on the topic of "Natural and applied sciences" (Mexico, 2022).

Name of the organization where the dissertation work was performed. The dissertation was completed at the Department of Genetics of Baku State University.

The structure and scope of the dissertation. The dissertation work consists of the introduction (17 096 symbols), 5 chapters (I chapter – 57 860 symbols, II chapter – 13 308 symbols, III chapter – 68 791 symbols, IV chapter – 37 704 symbols, V chapter – 3 872 symbols), conclusion (1 691 symbols), result (3 407 symbols), practical recommendations (1 319 symbols), list of literature with 210 titles (35779 symbols) and list of abbreviations (705 symbols), is

reflected on 150 computer pages, general volume (excluding schemes, figures, tables, graphs and list of literature) 167895 symbols. The dissertation includes 22 paragraphs, 30 figures, 9 schemes and 6 tables.

CHAPTER I. LITERATURE REVIEW

Chapter I of the dissertation is a summary, compiled on the basis of local and foreign literature. The chapter analyzes inherited metabolic disorder, their classification, molecular characteristics of galactosemia, biochemical and clinical polymorphism, frequency of occurrence in the world population, and other aspects in a comparative and logical manner, referring to literature sources.

CHAPTER II. MATERIAL AND METHODS OF THE RESEARCH

Screening for the galactosemia, inherited metabolic disorder was conducted in Baku during 2015-2023 using capillary and venous blood from 576 (299 boys and 277 girls) newborns and 138 (70 boys and 68 girls) children with suspected metabolic disorder (total 714 blood samples).

The activity of the galactose-1-phosphate uridylyltransferase enzyme in newborns was determined using the immunoenzyme assay (IEA) method[7].

Genomic DNA was extracted from lymphocytes using DNA kits, and amplification quality was determined by electrophoresis in 1.7% agarose gels for 45-60 min.

For PCR, a reaction mixture consisting of 5 μl of genomic DNA, 30 μl of special distilled water, 8.2 μl of 0.5M TAE buffer, 2.5 μl each of F (forward) and R (reverse) primers, 1.3 μl of dNTP, 0.63 μl of *Taq* DNA polymerase enzyme was prepared and PCRs were performed at 95°C for 2 minutes of denaturation, 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 77°C for 2 minutes, as well as a 10-minute at

[7] Perlmann, Peter et al. "Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. İmmunokimya. 1971; 8(9): 871–4.Perlmann, Peter et al. "Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G." İmmunokimya. 1971; 8 (9): 871–4.

72°C and completed at 4°C. The study used a100 bp (100 n.c.) *DNA Ladder*, and the amplicons were stained in 0.5 μg/ml ethidium bromide solution. Allelic variants of the gene were identified through screening of the nucleotide sequences of amplicons.

A regression analysis was conducted using the Maple computer program to determine the dependence of galactose-1-phosphate uridylyltransferase enzyme activity on the corresponding indicators of the mother and father in newborns and children with galactosemia.

CHAPTER III. BIOCHEMICAL AND MOLECULAR-GENETIC RESEARCH OF THE GALACTOSEMIA, INHERITED METABOLIC DISORDER

As a result of the study of the activity of the galactose-1-phosphate uridylyltransferase enzyme in newborns and children with suspected metabolic disorder, it was found that the enzyme was almost completely deficient in 4 newborns and 1 patient child, and partially synthesized in 1 newborn and 1 patient child (Table 1). Thus, the enzyme activity in newborn-1, newborn-2, newborn-4, and newborn-5 was determined to be 7 mU/g Hb, 8 mU/g Hb, 8 mU/g Hb and 7 mU/g Hb, respectively, which corresponds to a complete deficiency of the enzyme and a homozygous inheritance type. According to accepted international standards, the activity of the galactose-1-phosphate uridylyltransferase enzyme is 308 mU/g Hb in the norm, 140–222 mU/g Hb in the heterozygous type caused by missense mutations, 57–140 mU/g Hb in the Duarte variant, and ~ 8 mU/g Hb and lower in the homozygous type of the disorder. In newborn-3, the enzyme activity under study measured 90 mU/g Hb, corresponding to the inherited Duarte variant of galactosemia. Residual activity of the galactose-1-phosphate uridylyltransferase enzyme in the parents of the newborns confirmed their heterozygous carrier status; thus, in the parents of 5 newborns with enzyme activity below normal, the enzyme activity varied within the range of 150 - 222 mU/g Hb (Table 1).

According to the biochemical parameters of the blood of newborns with galactosemia and children with suspected metabolic disorder (Table 2), the amount of the glucose-6-phosphate dehydrogenase (G6PD) enzyme in the blood (normal 100%) was

within 10-30%, with the exception of newborn-3 (70%), which indicates that in newborn-3, the substrate of the G6PD enzyme is formed by the conversion of galactose monosaccharide to glucose monosaccharide and there is a need for the synthesis of this enzyme.

Table 1. Galactose-1-phosphate uridylyltransferase enzyme activity and inheritance type in patient newborns, children and family members

Patient	Galactose-1-phosphate uridylyltransferase enzyme activity	Genotype
Newborn-1	7 mU|g Hb	Compound
Mother – 1	170mU|gHb	Heterozygote
Father – 1	150 mU|gHb	Heterozygote
Newborn-2	8 mU|g Hb	Homozygote
Mother –2	170 mU|gHb	Heterozygote
Father – 2	180 mU|Hb	Heterozygote
Newborn-3	90 mU|g Hb	Homozygote
Mother – 3	222mU|g Hb	Heterozygote
Father – 3	210 mU|g Hb	Heterozygote
Newborn-4	8 mU|g Hb	Homozygote
Mother –4	210 mU|g Hb	Heterozygote
Father –4	170 mU|g Hb	Heterozygote
Newborn-5	7 mU|g Hb	Homozygote
Mother –5	222 mU|g Hb	Heterozygote
Father –5	190 mU|g Hb	Heterozygote
Patient child-1	150mU/g Hb	Heterozygote
Mother-patient child-1	170mU/g Hb	Heterozygote
Father-patient child-1	160mU/g Hb	Heterozygote
Patient child-2	11mU/g Hb	Homozygote
Mother-patient child-1	170mU/g Hb	Heterozygote
Father-patient child-1	160mU/g Hb	Heterozygote

Increased liver enzymes (ALT and AST) in newborns, except for newborn-3 (52 IU/L - 57 IU/L and 55 IU/L - 58 IU/L, respectively) may be explained by impaired galactose → glucose conversion and the toxic effect of accumulated galactose in the blood on the liver. The

highest level of this effect is observed in newborn-1. In patient 1 with suspected metabolic disorder, these indicators (42 IU/L and 45 IU/L, respectively) were almost within the normal range, while in patient 2, they increased significantly, reaching values of 97 IU/L and 63 IU/L, respectively.

Table 2. Biochemical parameters of blood in patients with galactosemia

Patients	Biochemical indicators
Newborn-1	Q6FD – 10-30% (100%)
	ALT – 57 IU/L (10-40 IU/I)
	AST – 58 IU/L (15-41 IU/L)
	Glucose – 1,9 mmol/l (4,1-5,9)
Newborn-2	Q6FD – 20% (100%)
	ALT – 57 IU/L (10-40 IU/I)
	AST – 55 IU/L (15-41 IU/L)
	Glucose – 1,7 mmol/l (4,1-5,9)
Newborn-3	Q6FD – 70% (100%)
	ALT – 43 IU/L (10-40 IU/I)
	AST – 45 IU/L (15-41 IU/L)
	Glucose – 3,5 mmol/l (4,1-5,9)
Newborn-4	Q6FD – 20% (100%)
	ALT – 53 IU/L (10-40 IU/I)
	AST – 57 IU/L (15-41 IU/L)
	Glucose – 2 mmol/l (4,1-5,9)
Newborn-5	Q6FD – 20% (100%)
	ALT – 52 IU/L (10-40 IU/I)
	AST – 56 IU/L (15-41 IU/L
	Glucose – 1,8 mmol/l (4,1-5,9)
Patient child-1	Q6FD – 20% (100%)
	ALT – 42 IU/L (10-40 IU/I)
	AST – 45 IU/L (15-41 IU/L)
	Glucose – 1.8 mmol/l (4,1-5,9)
Patient child-2	Q6FD – 20% (100%)
	ALT – 97 IU/L (10-40 IU/I)
	AST – 63 IU/L (15-41 IU/L)
	Glucose – 1.9 mmol/l (4,1-5,9)

The blood glucose level in newborns, except for newborn-3, varied within the range of 1.7 mmol/l (newborn-2) - 2 mmol/l (newborn-4), and in newborn-3 it approached the norm and amounted to 3.5 mmol/l. The blood glucose levels in children with suspected metabolic disorder were also low, with values of 1.8 mmol/l (patient 1) and 1.9 mmol/l (patient 2), respectively, once again confirming that galactose → glucose conversion was minimal in the patients studied.

Molecular genetic identification of the structure of the *GALT* gene in 5 newborns with altered galactose-1-phosphate uridylyltransferase enzyme activity, and 2 children with suspected metabolic disorder, as well as in 2 consanguineous married couples with unexplained infant death in their second-degree family members, revealed 10 different types of mutations: A→G transitions at positions c.563 and c.940, C→T transitions at positions c.652, c.974 and c.997, G→C transversion at position c.378-27 of the intron II, G→A transitions at positions c.507+62 and c.508-24 of the intron IV, c.129_ -126 deletion in front of exon I of the gene (in the upstream position) and c.-119_ -116 deletion near the promoter region (Table 3).

As a result of the identification of the nucleotide sequence of the *GALT* gene in newborn-1, it was observed that adenine nucleotide at position 563 of the gene in exon VI was replaced by the guanine nucleotide (A→G), and the cytosine nucleotide at position 974 of the gene in exon X was replaced by the thymine nucleotide (C→T). In the heterozygous case, the A→G transition at position c.563 (nucleotide 34648170 of chromosome 9 in the genome) is a missense mutation, causing the amino acid glycine to be replaced by the amino acid arginine (Gly→Arg) at position 188 in the amino acid sequence of protein. Since glycine is a non-polar hydrophobic amino acid and arginine has a positively charged side chain, the Gly→Arg substitution results in a change in the spatial conformation of the protein, resulting in the loss of its native structure and thus its activity.

In newborn-1, the transition of the cytosine nucleotide to the thymine nucleotide (C→T) at position 974 of the *GALT* gene (nucleotide 34649479 of chromosome 9 in genomic DNA) on homologous chromosomes causes the replacement (Pro→Leu) of the proline amino acid with the leucine amino acid at amino acid position 325 of the protein.

Table 3. Identified mutations in the *GALT* gene responsible for the galactose-1-phosphate uridylyltransferase enzyme

Patient	Types of mutations and the amino acid substitutions they cause	Genotype
Newborn-1	c.563 (A→G); p.188 (Gly→Arg) c.974 (C→T); p.325 (Pro→Leu)	Compound
Newborn-2	c.997 (C→T); p.333 (Arg→Trp)	Homozygote
Newborn-3	c.940 (A→G); p.314 (Asn→Asp) exon 1 upstream (c.129_ -126del) c.378-27 (G→C) - intron variant c.507+62 (G→A) - intron variant c.508-24 (G→A) - intron variant c.652 (C→T) p.218 (Leu=)	Homozygote Heterozygote Heterozygote Heterozygote Heterozygote Heterozygote
Newborn -4	c.563 (A→G) p.188 (Gly→Arg)	Homozygote
Newborn -5	c.563 (A→G) p.188 (Gly→Arg)	Homozygote
Patient child-1	c.563 (A→G) p.188 (Gly→Arg)	Heterozygote
Patient child-2	c.563 (A→G) p.188 (Gly→Arg)	Homozygote
Married couple 1- (male)	c.-119_ -116 del (Non-coding)	Heterozygote
Married couple 1- (female)	c.-119_ -116 del (Non-coding)	Heterozygote
Married couple-2 (male)	c.-119_ -116 del (Non-coding)	Heterozygote
Married couple-2 (female)	c.-119_ -116 del (Non-coding)	Heterozygote

The current nucleotide substitution is a missense mutation, detected in the heterozygous state, resulting in a conformational change and activity loss of the protein. Although proline and leucine belong to aliphatic amino acids with a branched side chain, proline has a cyclic structure and a secondary amino group, and always disrupts the alpha helical structure, while leucine, readily participates in the formation of both hydrogen and peptide bonds, and participates at the formation and stabilization of the alpha helical structure and hydrophobic protein core. This ultimately leads to disruption of the normal folding process of protein during gene expression and the inability to form the native structure of the enzyme that can function.

Thus, both missense mutations detected in newborn-1 are compound in nature and, despite being observed in a heterozygous state, result in a disruption of gene expression of each allele variant at the folding level, loss of enzyme activity in the body, and an enzyme

deficiency equal to 7 mU/g Hb.

Examination of table 3 reveals that the A→G transition identified in the heterozygous state at position 563 of the gene in exon VI in newborn-1 is characteristic for the Azerbaijani population, and is observed in the homozygous state in newborn-4 and newborn-5, children of the same family, which gives reason to note that this mutation is also characteristic for their parents. A review of the literature shows that the A→G transition observedat position 563 of the *GALT* gene accounts for 60–70% of classical galactosemia alleles in the Caucasian population, 54–70% in the US population, and 0.29% in the Slovenian population[8]. In the group of newborns from Baku, the frequency of the null allele caused by the transition c.563 (A→G) was 0.434%, in the group of children with suspected metabolic disorder - 1.087%, among newborns and children with suspected metabolic disorder - 0.56%.

Molecular genetic analysis of the *GALT* gene in newborn-2 revealed a homozygous substitution of the cytosine with the thymine nucleotide (C→T) at position 997 of the gene (Figure 1). This transition located at nucleotide 34649502 on chromosome 9 in the genomic DNA, is a missense mutation, resulting in the substitution (p. 333 (Arg→Trp)) of the arginine amino acid with tryptophan amino acid at amino acid position 333 of the protein. Arginine is a polar, positively charged amino acid, whereas tryptophan is non-polar and hydrophobic. Therefore, the Arg→Trp substitution resulting from the C→T transition leads to a significant change in the protein's spatial conformation, ultimately causing a loss of function.

As a result of the identification of the nucleotide sequencing of the *GALT* gene in newborn-3, six different mutations were identified (Table 3). However, the patient phenotypically exhibited clinical features consistent with the mild Duarte variant of galactosemia (tables 1 and 2). Three of the detected mutations were SNPs (single nucleotide polymorphisms) located in the intronic regions of the gene.

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[7] Demirbas, D. Hereditary galactosemia. Metabolism / D.Demirbas, A.I.Coelho, M.E.Rubio-Gozalbo [et al.] // 2018; 83:188–196. doi: 10.1016/j.metabol.2018.01.025. *[DOI] [PubMed] [Google Scholar]*

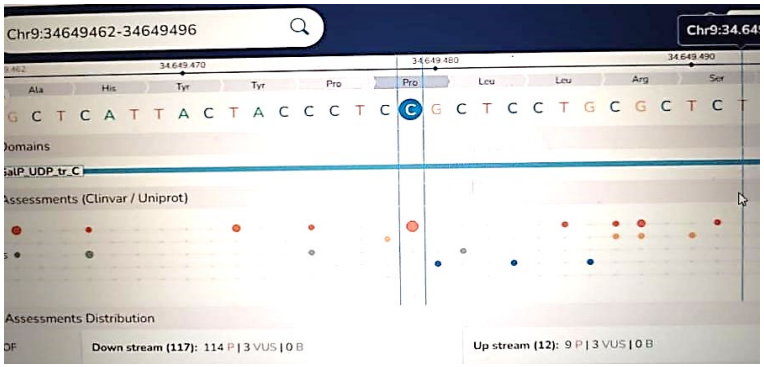

Figure 1. C→T transition detected at position c.997 of the *GALT* gene

These did not result in any changes to gene expression or enzyme structure and are therefore considered neutral mutations. One of these is a G→C transversion at position c.378-27 of the gene, i.e., 27 nucleotides before nucleotide 378 of the *GALT* gene - nucleotide 1 of exon III, in intron II (Figure 2). Its cytogenetic location is the subsegment 3 of segment 13 of the small arm of chromosome 9, and its genomic position is nucleotide 34647805 of chromosome 9. The inheritance type is heterozygous.

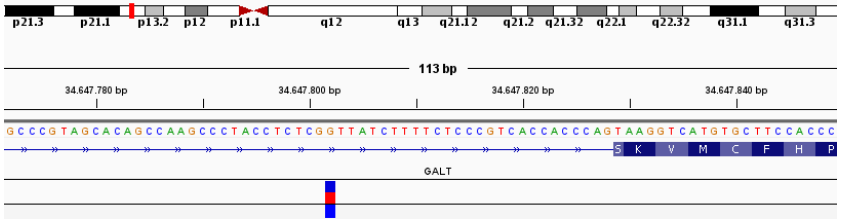

Figure 2. G→C transition detected at position c.378-27 of the *GALT* gene

Another neutral mutation is the G→A polymorphism at position c.507+62 of the gene, occurring 62 nucleotides after from nucleotide 507 of exon IV, within intron IV (Figure 3). It was detected in a heterozygous state. Its cytogenetic location is the subsegment 3 of segment 13 of the small arm of chromosome 9, and its genomic position is nucleotide 34648023 of chromosome 9.

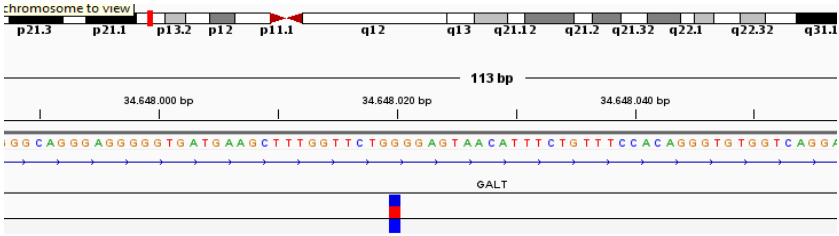

Figure 3. G→A transition detected at position c.507+62 of the *GALT* gene

In newborn-3, the neutral mutation III is a G→A transition that occurred in the heterozygous state at position c.508-24 of the *GALT* gene, i.e. 24 nucleotides before nucleotide 508 (exon V), in intron IV (Figure 4). Its cytogenetic location is the subsegment 3 of segment 13 of the small arm of chromosome 9, and its genomic position is nucleotide 34648091 of chromosome 9.

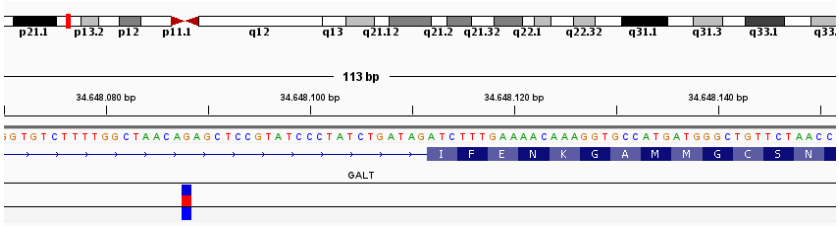

Figure 4. G→A transition detected at position c.508-24 of the *GALT* gene

In newborn-3, in addition to the neutral nucleotide polymorphisms detected in the intronic regions of the *GALT* gene, two nucleotide substitutions were also identified in the exonic regions. Thus, one of them is the C→T transition that occurred in exon VII, at position c.652 of the gene (nucleotide 34648421of chromosome 9) (Figure 5), which, although detected in the heterozygous state, was a silent mutation, resulting in the creation of a synonymous codon and the retention of the leucine amino acid at amino acid position 218 of the protein, and did not affect gene expression or the activity of the enzyme in the body.

Another SNP is an A→G transition in exon X of the gene, at position c.940 (nucleotide 34649445 of chromosome 9) (Figure 6),

which causes the AAC codon to be replaced by the GAC codon, thereby resulting in the substitution of the amino acid asparagine for asparagine acid at amino acid position 314 of the protein (Asn→Asp). While Asn is a neutral, hydrophilic amide, Asp is a negatively charged, acidic amino acid. Since the Asn→Asp substitution at position 314 in the protein structure occurs in the interdomain region, it partially affects the folding of the protein and, in the homozygous case, leads to a relative decrease in enzyme activity (90 mU/g Hb) and phenotypically to the emergence of the Duarte - clinically mild form of the disorder. In this regard, the c.940 (A→G) transition, also known as the "Los Angeles (LA) phenotype," can be considered a leaky mutation.

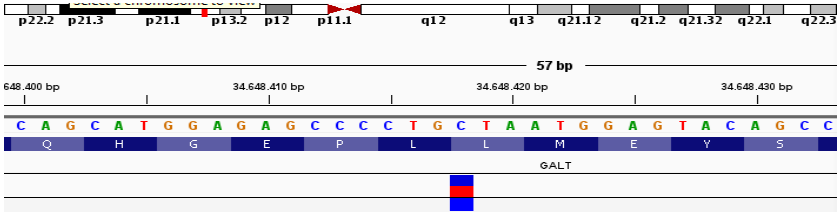

Figure 5. C→T transition detected at position c.652 of the *GALT* gene

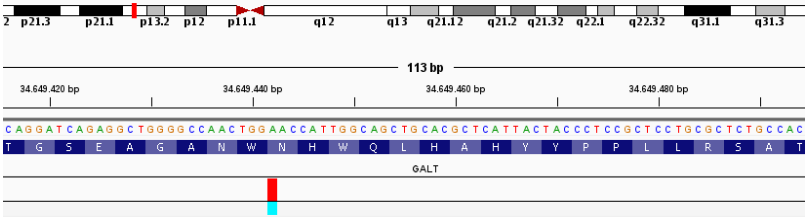

Figure 6. A→G transition detected at position c.940 of the *GALT* gene

Finally, the last mutation detected in the *GALT* gene in newborn-3 was a deletion of the CAGT nucleotides at positions c.129-126 of the gene (Figure 7). The CAGT deletion at positions c.129-126 occurs 129 nucleotides before exon I, 9 nucleotides further from the promoter, in the 5'UTR upstream area, and is a deficiency of nucleotides 34646572-34646576 of chromosome 9. The change occurred not in the entire gene, but in a part close to the gene. Although this deficiency, which

does not cause any changes in the protein structure, is suspected to have an effect on protein expression and thus pathogenic significance in some sources (e.g., ClinVar, NCBI gene databases), in our study, screening in the heterozygous state, along with other mutation variants in newborn-3, and the fact that the protein activity of 90 mU/g Hb is related to the A→G transition at position c.940 detected in the homozygous state, give reason to say that the current mutation does not play a role in gene expression.

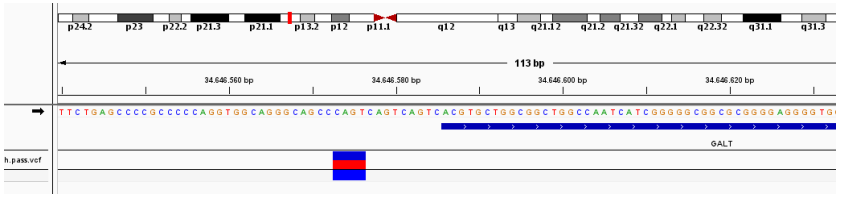

Figure 7. CAGT deletion at position 129-126 of the *GALT* gene

Nucleotide sequence screening of the *GALT* gene in 2 children with suspected metabolic disorder resulted in the identification of the same mutation - an A→G transition at position c.563 - detected in newborn-1, newborn-4, and newborn-5. The c.563 (A→G) transition was heterozygous in patient child-1, causing an enzyme activity of half - 150 mU/g Hb, while in patient child-2, it was detected in a homozygous state, resulting in enzyme deficiency (11 mU/g Hb). Although average values of enzyme activity (170 mU/g Hb in the mother, 160 mU/g Hb in the father) were also observed in the parents of patient child-1, only 1 mutation was detected in the child in a heterozygous state, which indicates that the mutant allele was inherited from only one of the parents to the child. In patient child-1, both X-linked mycopolysaccharidosis type II, caused by the deletion of nucleotides 1215-1217 in exon 11 of the *IDS* gene (occuring of enzyme deficiency due to the deletion of phenylalanine at position 508 in the iduronate-2-sulfotase enzyme), and the null allele (c.1834 (C→T); p.612 (Arg→Trp)) of the *ABCC2* gene responsible for the MRP2 protein, which is involved in the detoxification of metabolic products of a number of drugs, were detected in the heterozygous state, which causes the manifestation of severe clinical symptoms in the patient.

Molecular genetic screening of the *GALT* gene in two

consanguineous married couples involved in the study revealed that each of them had the same mutation in a heterozygous state - a deletion of GTCA nucleotides (nucleotides 34646576-34646579 of chromosome 9) at positions c.-119_-116 in the 5' untranslated region, 119 nucleotides before exon I of the gene, 9 nucleotides further from promoter (Figure 8).

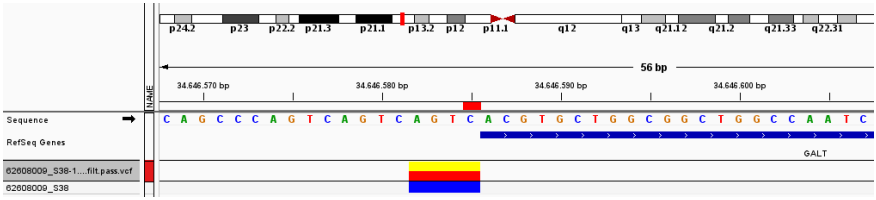

Figure 8. *GALT* gene c.-119_-116 (GTCA) deletion

Although the mutation occurs in the 5'UTR, not within the gene, it affects promoter activity and thus protein expression, and the literature shows that this mutation reduces the expression level of GALT gene mRNA by 55%[3].

Thus, the results of the immunoenzymatic analysis and molecular-genetic screening of the *GALT* gene confirmed the presence of galactosemia among newborns and patient children in Azerbaijan, made it possible to identify various null and isoalleles of the disorder found in the world population, as well as determine their occurence frequencies.

The identification of the disorder in 5 out of 576 newborns screened, and in 2 out of 138 children with suspected metabolic disorder, gives reason to say that it occurs with a frequency of 0.86% among newborns (Figure 9), 1.45% among patient children (Figure 10), and 0.98% among the total number of people studied (714 people).

The incidence of newborn galactosemia patients among the total studied people was 0.70% (Figure 11).

The higher frequency of classical galactosemia in newborns in Baku (0.0086 in 2015-2023, 0.00097 per year) compared to the relatively high frequency of European (1:40,000-1:60,000 or 0.000025-0.000017) and US (1:50,000 or 0.00002) populations[4] suggests the necessity of studying this disorder in the Azerbaijani population, especially in cases of consanguineous marriages, and the

importance of both early and prenatal diagnostics through molecular genetic screening.

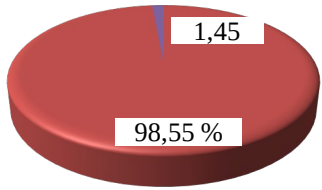

Figure 9. The proportion of newborns with galactose-1-phosphate uridylyltransferase enzyme - deficiency among all newborns (0.86%).

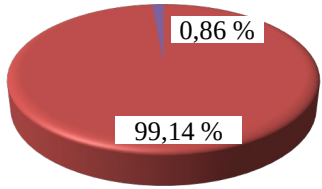

Figure 10. The proportion of patients with galactose-1-phosphate uridylyltransferase enzyme deficiency among children with suspected metabolic disorder (the incidence of the disorder is 1.45%)

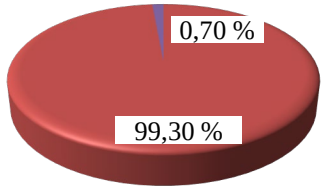

Figure 11. The proportion of newborns with a deficiency of the galactose-1-phosphate uridylyltransferase enzyme among newborns and children with suspected metabolic disorder (incidence of the disorder - 0.70%)

CHAPTER IV. PREVENTION OF GALACTOSEMIA DISORDER

It is recommended to use both types of medical-genetic counseling for the prevention of galactosemia. Based on prospective medical-genetic counseling, proper pregnancy planning should be achieved in families at genetic risk and the probability of having a patient child should be minimized, while retrospective medical-genetic counseling should be applied to families with patient children.

In cases of risk of inherited disorder in close relatives or cases of death of newborns for an unknown reason, as well as in consanguineous marriages, future parents should seek medical genetic consultation before planning a pregnancy and, as prescribed by a geneticist, undergo molecular genetic screening of the *GALT* gene to determine whether they are carriage or not. In families included in the

genetic risk group based on medical-genetic consultation, as well as in families where a patient child was born, prenatal diagnosis of the fetus in the womb should be performed during early pregnancy in order to prevent the birth of another patient child.

The simplicity of obtaining chorionic cells, the detection of the disorder in earlier stages of pregnancy, and the minimal risk of fetal infection when obtaining chorionic cells via the less invasive transcervical approach make transcervical biopsy optimal for examining *GALT* gene mutations at the DNA molecule level.

CHAPTER V. REGRESSION ANALYSIS OF GALACTOSEMIA, INHERITED METABOLIC DISORDER IN NEWBORNS IN BAKU

5.1. Constructing a regression equation and calculation of R^2 determination coefficient. Regression statistical analysis of galactose-1-phosphate uridylyltransferase enzyme indicators in newborns, children with galactosemia and their parents was performed, the dependence of the *GALT* gene activity indicators (X) of the patients on the corresponding indicators in the mothers (Y) and fathers (Z) was investigated, and graphic descriptions expressing these dependencies were obtained based on the relevant equations.

5.2. Power regression models of the dependence of *GALT*gene indicators in newborns on the corresponding indicators of the mother and father. According to the graph of the dependence of *GALT* gene indicators on the corresponding indicators of mothers in newborn and children (Figure 12), when the activity of the *GALT* gene in mothers varied between 150-250, the power regression model $X = 6{,}279 \times Y^{0{,}1935}$ was equal to $R^2=0.8453$, which indicates a high adequacy of the model (adjusted regression coefficient $R^2=0{,}7835$).

According to the graph of the dependence of *GALT* gene indicators in newborn patients and children on the corresponding indicators of the fathers (Figure 13), when the activity of the *GALT* gene in the fathers varied between 150-250, $R^2=0{,}8690$ was obtained for the power regression model $X=6{,}92\cdot10^{-10}\cdot Z^{4.6369}$ (adjusted regression coefficient $R^2=8166$).

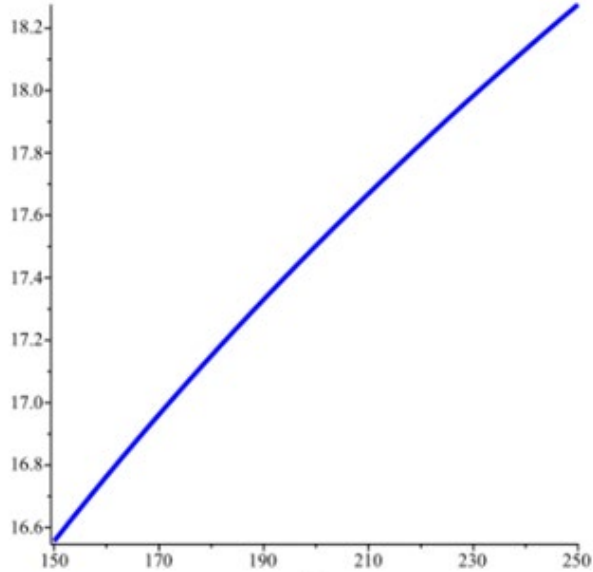

Figure 12. The dependence graph of *GALT* gene indicators on the corresponding indicators of mothers in patient newborns and children.

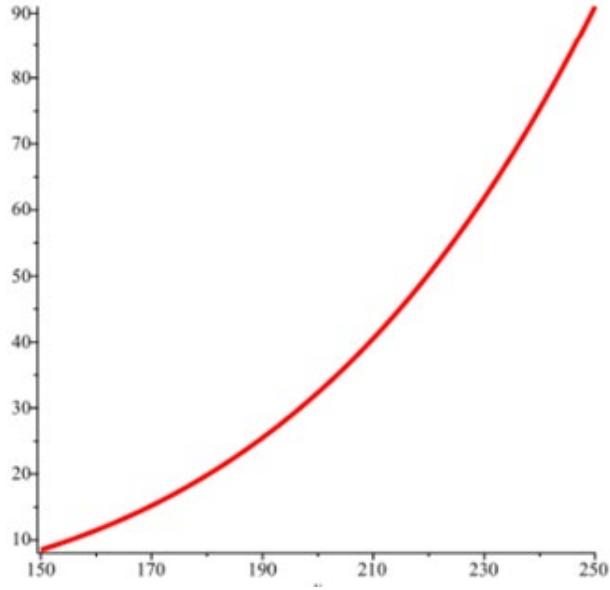

Figure 13. The dependence graph of GALT gene indicators on the corresponding indicators of fathers in patient newborns and children

5.3. The dependence of *GALT* gene indicators on the corresponding indicators of mothers and fathers in patient newborns and children. Based on the activity indicators of the *GALT* gene, a 2-variable nonlinear regression model was developed to determine the dependence of the indicators (X) of patient newborns and children on the indicators of the mother (Y) and father (Z) (Figure 14).

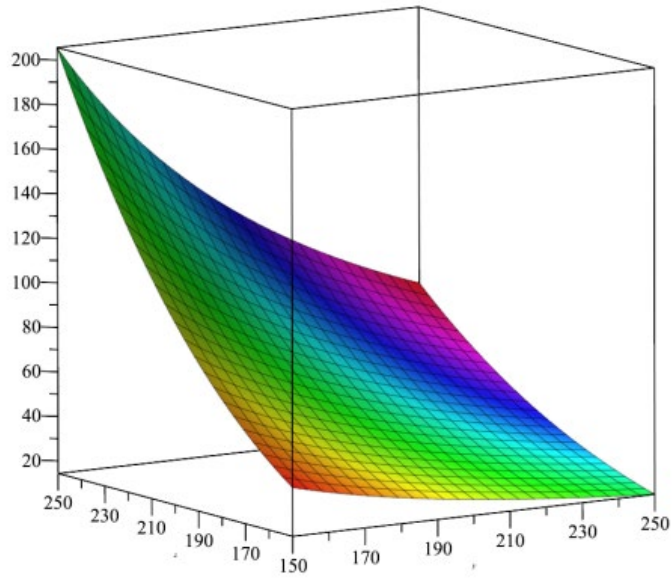

Figure 14. The dependence of *GALT* gene indicators on the corresponding indicators of both mothers and fathers in patient newborns and children

The equation of a multivariate nonlinear regression model is as follows: $X = 0.01270 \times Z^{3.4010} \times Y^{-1.8134}$. The model predicts that as the father's enzyme activity indicators increase, these indicators will also rise significantly in his children. In contrast, an inverse relationship is observed between the corresponding indicators of the mother and those of the child. The colored surface in the graph depicts the detected result of the model: at the top of the surface, Z is high, Y is low, X takes maximum values, and vice versa, as Y increases, X decreases sharply. This indicates the presence of a positive effect of factor Z (enzyme activity in the father) on X (patient child), and a negative effect of factor

Y (indicators of enzyme activity in the mother).

The correlation matrix (Table 4) compiled based on the regression model confirms that as the independent variable Z increases, the dependent variable X increases, and as the independent variable Y increases, X decreases.

Table 4. Correlation matrix of variables Y, Z and X

Variables	Y	Z	X
Y	1	0.765	-0.058
Z	0.765	1	0.122
X	-0.058	0.122	1

Thus, the applied multivariate non-linear regression model allowed to reveal that the variable X (the child's studied indicators) is more strongly associated with factor Z (the corresponding indicators of the father), while the effect of Y (the corresponding indicators of the mother) on Z was found to be low and negative**.** This also indicates the direct dependence of galactose-1-phosphate uridylyltransferase enzyme activity in children on paternal heredity, with the presence of positive correlations between them. The detected effect of maternal heredity on galactose-1-phosphate uridylyltransferase enzyme activity may also be due to epigenetic regulation of the activities of the corresponding maternal heredity units in the genome of the offspring.

RESULTS

1. Screening for the galactosemia, inherited disorder among newborns and children with suspected inherited metabolic disorder revealed heterozygous, homozygous, and compound inheritance types of galactose-1-phosphate uridylyltransferase enzyme deficiency.

2. The frequency of occurence of galactosemia caused by mutations in the *GALT* gene was 0.86% among newborns, 1.45% among patient children, and 0.98% in the general study group.

3. Among newborns, 9 different mutations were detected as a result of nucleotide sequencing screening of the *GALT* gene; one of them was a silent mutation (c.652 (C→T) p.218 (Leu=)), another was a c.129_ -126 deletion located in the untranslated region before exon 1 of the gene, and 3 were traced to intronic regions (c.378-27 (G→C); c.507+62 (G→A); c.508-24 (G→A)) and were not phenotypically detected and formed isoallelic variants of the gene. Four missense mutations, transitions in different exons (c.563 (A→G); c.940 (A→G); c.974 (C→T); and c.997 (C→T)), resulted in the formation of null alleles that disrupted the spatial conformation of the protein.

4. As a result of screening of the nucleotide sequencing of the *GALT* gene in 2 children with suspected metabolic disorder, the same missense mutation caused by an A→G transition, was identified at 563 position in exon VI in heterozygous and homozygous states, which caused a Gly→Arg substitution (p.188) the protein, resulting in a disruption of the folding process.

5. In patient child-1 the c.563 (A→G) transition was detected in the heterozygous state. X-linked mucopolysaccharidosis type II, caused by the deletion of nucleotides 1215-1217 in exon 11 of the *IDS* gene, and a null allele (c.1834 (C→T); p.612 (Arg→Trp)) of the *ABCC2* gene, responsible for the MRP2 protein involved to the detoxification of metabolic products of a number of drugs, were identified in the heterozygous state, which resulted in a sharp exacerbation of the clinical symptoms of galactosemia.

6. The frequency of the null allele, caused by the relatively high-intensity c.563 (A→G) transition of the *GALT* gene, was 0.434% in the newborn study group, 1.087% in the group of children with

suspected metabolic disorder, and 0.56% among newborns and children with suspected metabolic disorder.

7. Molecular genetic screening of the *GALT* gene in each member of two consanguineous married couples revealed the same mutation in the heterozygous state - a deletion of GTCA nucleotides at positions c.-119_-116 in the 5' untranslated region, 119 nucleotides before exon I of the gene, 9 nucleotides further from the promoter.

8. Polymorphisms of the *MCM6* enhancer of the *LCT* gene that could cause hypolactosis have not been detected in newborns with galactosemia and patients with suspected inherited metabolic disorder.

9. A 2-variable regression model of galactose-1-phosphate uridylyltransferase enzyme activity in patient newborns, children, and their parents showed a direct dependence of enzyme activity on paternal heredity, with statistically significant positive correlations between them.

PRACTICAL RECOMMENDATIONS

1. It is recommended that total galactose amount determination be mandatory included in the inherited metabolic screening of newborns (tandem MS), which is performed by heel testing in the first three days of life in both public and private hospitals.

2. It is recommended that neonatologists and geneticists participate in specialized training sessions for early screening of galactosemia in newborns and provide them with proper treatment from the first days of life.

3. If a neonatologist detects a deficiency of galactose-1-phosphate uridylyltransferase enzyme activity in newborns, prenatal diagnosis of the fetus in the womb during subsequent pregnancies is mandatory, taking into account the reproductive age of the parents.

4. Determination of galactose-1-phosphate uridylyltransferase enzyme activity by immunoenzyme analysis is recommended in patients hospitalized in Republic children's hospitals with a clinical picture similar to metabolic syndrome.

5. In families at genetic risk, it is recommended that parents undergo molecular genetic screening of the *GALT* gene through prenatal diagnosis before planning pregnancy, and in families where a patient child is born, during early pregnancy.

List of published scientific works related to the dissertation topic

1. Hacıyeva N.M., Əliyeva K. Ə., Hüseynova L.S., Qalaktoza-1-fosfaturidiltransferaza fermentinin geninin (GAL-1) molekulyar-genetik tədqiqi // AMEA Mikrobiologiya İnstitutunun elmi əsərləri. -Bakı: -2017, №1, - s.180-186
2. Hacıyeva N.M., Əliyeva K. Ə., Hüseynova L.S., İdentification of genetic mutations in the newborn with galactosemia combined maple syrup urine disease // Bakı Universitetinin xəbərləri. Təbiət elmləri seriyası. -Bakı: -2017, №2, -səh. 50-56
3. Hacıyeva N.M., Əliyeva K. Ə., Hüseynova L.S., Laktaza və qalaktoza-1-fosfat uridiltransferaza fermentlərinin genetik heterogenliyinin tədqiqi //AMEA Genetik Ehtiyatlar İnstitutunun elmi əsərləri. -Bakı: -2017, №1-2, -s. 188-191
4. Hacıyeva N.M. Ağcaqayın şirəsi adlı irsi mübadilə xəstəliyinin genetik diaqnostikası / Bakı Dövlət Universiteti Azərbaycan xalqının böyük oğlu, Ulu Öndər Heydər Əliyevin anadan olmasının 94-cü ildönümünə həsr olunmuş gənc alim və tədqiqatçıların elmi konfransının materialları (27-28 aprel), - Bakı: -2017, -səh. 92-93
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6. Hacıyeva N.M., Əliyeva K.Ə., Hüseynova L.S., Azərbaycan respublikasının əhalisində CFTR və GALT1 genlərinin mutasiyaları // Bakı Universitetinin xəbərləri. Təbiət elmləri seriyası. -Bakı:-2018, №3, -s. 51-57
7. Hacıyeva N.M. Hipolaktoziya xəstəliyində MCM6 geninin molekulyar genetik tədqiqi // Gəncə Dövlət Universiteti Fundamental, humanitar və təbiət elmləri seriyası. Elmi xəbərlər. -Gəncə: -2018, №4, -s.19-22
8. Hacıyeva N.M. Qalaktozemiya irsi xəstəliyinin molekulyar-genetik tədqiqi və profilaktikası // Naxçıvan Dövlət Universiteti Təbiət və tibb elmləri seriyası. Elmi əsərlər. -Naxçıvan: -2018, №7 (96), -səh. 246-251
9. Hacıyeva N.M. Qalaktozemiya irsi xəstəliyi aşkar edilmiş

yenidoğulmuşlarda biokimyəvi və molekulyar-genetik göstəricilərin tədqiqi / Azərbaycan Dövlət Pedoqoji Universiteti Doktorantların və gənc tədqiqatçıların XXII Respublika Elmi Konfransının materialları. Azərbaycan Xalq Cümhuriyyətinin 100 illik yubileyinə həsr olunur. -Bakı:-2018, №1, -s.112

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11. Hacıyeva N.M., Hüseynova L.S. Qalaktozemiya metabolik irsi xəstəliyinin genetik formaları /Bakı Dövlət Universiteti Ümummilli lider Heydər Əliyevin anadan olmasının 95 illiyinə həsr olunmuş VII Respublika elmi konfransının materialları. -Bakı: -2018, -s. 210-211
12. Hacıyeva N.M. Qalaktozemiya irsi mübadilə xəstəliyində GALT geninin molekulyar tədqiqi // Azərbaycan Memarlıq və İnşaat Universiteti Doktorantların və Gənc Tədqiqatçıların XXIII Respublika Elmi Konfdransının materialları. Böyük Azərbaycan şairi İmadəddin Nəsiminin 650 illik yubileyinə həsr olunur. -Bakı: -2018, №4, - s.74-75
13. Hajıyeva N.M. Mutation of duarte version of galactosemia (GALT) disease found in Azerbaijan // Bulletin of Science and Practice. -Moskva: -2022, №4, - p. 284-291 http://doi.org/10.33619/2414-2948/82/32
14. Hajıyeva N.M., Aliyeva K.A. Molecular heterogeneity of classical and duarte galactosemia // Bulletin of Science and Practice. -Moskva: -2022, №8, -p. 129-134 http://doi.org/10.33619/2414-2948/81/16
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The defense will be held on 30 June 2025 at 11:00 at the meeting of the Dissertation council FD 1.37 of Supreme Attestation Commission under the President of the Republic of Azerbaijan operating at Genetic Resources Institute of Ministry of Science and Education Republic of Azerbaijan

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The dissertation is accessible at the library of Genetic Resources Institute of Ministry of Science and Education Republic of Azerbaijan

Electronic versions of the dissertation and its abstract are available on the official website of the Genetic Resources Institute of Ministry of Science and Education Republic of Azerbaijan (https:www.genres.az).

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