Texila Advanced Journal of Multidisciplinary Health Research
DOI: 10.21522./TAJMHR.2016.05.01.Art010

The Role of miRNA Molecules and DNA-Methylation on Expression of Circadian Rhythm Genes in Kids with Mental Disorders

Zahra J. Ramadan, Owayes M. Hamed*, Elham kh. Abdullah
Department of Biology, College of Science, University of Mosul, Mosul, Iraq & Collage of medicine, University of Ninevah, Mosul, Iraq

Abstract

The variation of circadian rhythm genes is involved in mental disorders in kids, and the disrubance of circadian rhythm considered as a risk factor to the development of most mental disorders. Our aim was to study the estimated the role of miRNA molecules(miRNA24, miRNA449a, and miRNA102 on regulated for Per1, Per2, and Per3) and DNA methylation on gene expression of circadian clock genes .The study sample comprised of 40 patient of kids their ages range between (2 – 12 years) having mental disorders , and 20 sample of healthy kids as a control . The study topics were divided into 3 sections: first, determining the level of gene expression for Per1, Per2, and Per3 genes using the qRT-PCR technique. Second section: determination of the level of Non-coding RNA molecules that regulate Per family genes using the qRT-PCR technique. The third section measures the percentage of methylation in the promoter of Per family genes using the PCR technique. The results of the first section showed a significant decrease in the Per1 *gene expression; it was the value of folding expression cDNA* Per1 *gene = 0.33, and a significant increase in the* Per2 *and* Per3 *genes; it was the value of folding expression cDNA* Per2 *gene = 1.76 and folding expression cDNA* Per3 *gene = 1.58. The results of the second section showed a significant decrease in miRNA gene expression; the value of folding expression cDNA miRNA24* Per1 *gene = 0.41, foldin expression cDNA miRNA449a* Per2 *gene = 0.20, and folding expression cDNA miRNA102* Per3 *gene = 0.56. The results of the third section of the DNA methylation showed that DNA methylation in promoter of the Per1 gene was present in 50% of the samples, in the promoter of the Per2 gene, in 43% of the samples, and in the promoter of the Per3 gene, in 0% of the samples.*

Keywords: *Circadian Rhythum Gene, Gene Expression, Methylation, Mirna, Neurodevelopmental Disorders, q-PCR.*

Introduction

Almost all species have internal timekeeping systems called circadian rhythms, which are essential for preserving homeostasis and fostering survival in the face of environmental change. The circadian clock, an internal oscillator that synchronizes a range of behavioral, physiological functions, and biochemical processes with daily cycles, controls these rhythms. Numerous ailments, including mental disorders and insomnia, are linked to disruptions in the circadian cycle [1]. Evolutionarily conserved across species, the circadian clock is produced by cell-autonomous transcriptional/translational feedback loops (TTFLs). Mammals have a major TTFL that has two directions: positive and negative. In the positive direction, BMAL1 and CLOCK form a heterodimer and bind to E-box areas on clock-controlled genes (CCGs), such as Periods (Per genes) [2]. As PER levels increase in the cytoplasm, they go to the nucleus and interact with the CLOCK: BMAL1 dimer to decrease CCG transcription through either displacement or blocking

Received: 27.03.2025 ***Accepted: 06.05.2025*** ***Published on: 29.05.2025***
****Corresponding Author: owsbio31@uomosul.edu.iq***

processes, depending on PER levels. There are perhaps nine circadian rhythm genes; Period family genes which contain *PER1*, *PER2* and *PER3* gene, Cryptochrome family genes which contain *Cry1* and *Cry2* gene. Also the core of circadian rhythm genes *BMAL1* and *CLOCK* genes, Also there are other genes like *TIMELESS* and *CASEIN KINASE* 1e genes, all this genes are control the human circadian rhythm [3]. The three period (PER) genes produce unique proteins with the PER-ARNT-SIM (PAS) domain that are mostly involved in nucleocytoplasmic function rather than direct DNA binding. helix-loop-helix transcription factors (PAS) are encoded by the CLOCK and BMAL1 genes [4]. A molecular clockwork made up of interconnected feedback loops for gene expression is created from the products of these genes. The PER genes play a crucial role in the auto-regulatory transcription and translation feedback loops of the circadian genes, which form the basis of the current concept of these oscillators. The TTFL's negative arm is made up of these interactions [5]. Additionally, the accurate creation and control of circadian rhythms in mammals depends on auxiliary feedback loops. Major health risks and mental issues can result from disruptions in this internal cycle. According to reports, sleep-related disorders affect at least 50% of global population [6]. An internal time-keeping system called the circadian clock regulates many physiological functions through the production of circadian rhythms in gene expression, which are then converted into cycles in metabolism and behavior. The SCN in the brain has the central clock, and other tissues and organ systems contain peripheral clocks, which together comprise the system [7]. The RNA World theory states that most of the information processing and metabolic changes required for biology to diverge from chemistry in the early history of life were performed by RNA or RNA-like substances [8]. RNA serves as a "photocopier" of DNA, a building block of proteins, a structural element of ribosomes and ribozymes, and a regulator of several biological processes, among other functions [9]. Thus, RNA may have been the precursor of life and evolved alongside it. Given the notion of RNA World and the assumed origin of the circadian clock in the first living species on Earth, it is logical to surmise that the RNA regulation system and the circadian system coevolved and maybe interacted with one another. It has long been believed that non-coding RNAs that are unable to produce proteins are all "junk," but more recent research has challenged this notion [10].

Non-coding RNAs are, in fact, a popular subject in science right now. Numerous non-coding RNAs play an important role in biological systems and use different strategies to make up for their inability to be translated into proteins [11]. A class of non-coding RNAs known as microRNAs (miRNAs) serves as a post-transcriptional regulator. MiRNAs are involved in the regulation of multiple elements that are critical to the body's basic systems [12]. Recent data indicates that miRNAs control the circadian rhythm of gene expression and vice versa. Post-transcriptional and post-translational regulation, which is predicated on a well-functioning transcription and translation feedback system, accurately controls the molecular mechanism of the circadian clock [13]. Nonetheless, it has been proposed that a number of illnesses in the body are caused by the disruption of the circadian clock system. If there is a malfunction in the central nervous system, it may lead to neurological disorders such as sleep disorders, neurodegenerative diseases and Neurodevelopmental disorders (NDDs) which include Attention deficiency and hyperactivity disorder (ADHD), autism spectrum disorder (ASD) and Tourette syndrome (TS) [14 - 15]. Furthermore, patients with neurodevelopmental disorder, neurodegenerative diseases, and sleep disorders frequently have miRNAs that are improperly expressed in several tissues, the

blood, and/or bodily fluids in common. These data suggest that miRNAs may serve as useful therapies as well as biomarkers of disease etiology due to their capacity to alter the expression of genes linked to or causative of diseases [15-22].

Materials and Methods

Case study

The current study included (40) male children have mental disorders And (20) healthy male children who do not suffer from any disorder and in the same age group, ranging from (2-12) years, who visited the "Rufaidah Medical Clinic" in a period of time for (3 months) , based on approval form of a research protocol/ ministry of health and environment (form number 02/2024).

Blood sample collection and storage: (5.0) ml of venous blood was drawn from children with mental disorders and was divided into two parts:

1. The first part was placed in 1.5 ml eppendorf tubes pre-filled with 750 µl trizol for mRNA and non-coding RNA extraction.
2. The second part was placed in tubes containing an anticoagulant EDTA for DNA extraction for DNA Methylation test.

The gene expression level of *Per1, Per2 and Per3 genes and* Non-coding RNA was analyzed depend on q-PCR technique and the process includes several steps:

*RNA and non-coding RNA Extraction***:** After mixing 250 ml of blood sample with 750 ml of Trizol solution, an RNA extraction kit supplied by Transgenbiotech company is used, then Nano drop device use to measurement the purity of extract RNA.

*The process of converting the extracted mRNA and Non-coding RNA molecule into a cDNA***:** After the extraction of mRNA and Non-coding RNA is completed, it is converted into cDNA dependence on the reverse transcriptase enzyme activity, by used the Transgenbiotech company kit.

RT PCR reaction: specific housekeeping genes with the primer of genes used to:

Detection the Levels of Gene Expression for mRNA for PER Genes

The sequence of primers use in RT-PCR reaction for mRNA molecules was *Per1*- RT Forward GGACACTCCTGCGACCAGGTACTG, *Per1*- RT Reversed GGCAGAGAGGCCACCACGGAT, *Per2*- RT Forward CGCAGGGTGCGCTCGTTTGA, *Per2*- RT Reversed GGGCTCTGGAACGAAGCTTTCG, *Per3*- RT Forward GGTCGGGCATAAGCCAATG, *Per3*- RT Reversed GTGTTTAAATTCTTCCGAGGTCAAA, *Housekeeping*- RT Forward TGACCCAGATCATGTTTGAG, *Housekeeping*-RT Reversed CGTACAGGGATAGCACAG [16]. The component of reaction with final reaction volume 20µl was Ultra sybr q-PCRMIX with 10 µl volume, forward primer RT with 0.5 µl volume, Reversed primer RT with 0.5 µl volume, template of cDNA with 4 µl volume and distilled water with 5 µl volume. The program of RT-PCR for mRNA molecules was four stages; Pre denaturation at temperature 95°C for time period 10 min, and the Denaturation at temperature 95°C for 15 sec, Annealing /Extension at 60°C for 1 min and Melting curve analysis at 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec.

Detection the Gene Expression Levels of miRNA Molecules

The sequence of primers use in RT-PCR reaction for miRNA molecules was miRNA 24 –F regulate Per1 Gene AACACGTGTGCCTACTGAGCT, miRNA 449a-F regulate Per2 Gene AACACGCTGGCAGTGTATTGTTAG,

miRNA 103–F regulate Per3 Gene AACAAGAGCAGCATTGTACAGGG, universal-R for all genes CAGTGCAGGGTCCGAGGT, U6 –F for housekeeping GTGCTCGCTTCGGCAGCA and U6 –R for housekeeping CAAAATATGGAACGCTTC [16]. The component of reaction with final reaction volume 20µl, the Ultra syber q-PCR master mix was first component with 10 µl volume, forward primer RT with 0.5 µl volume, Reversed primer RT with 0.5µl volume, the template of cDNA with 4 µl volume and distilled water with 5 µl volume. The RT-PCR program for miRNA molecules was four stages; Pre denaturation at temperature 95°C for time period 10 min, Denaturation at temperature 95°C for time period 15 sec, Annealing /Extension at temperature 60°C for time period 1 min and Melting curve analysis at temperature 95°C for time period 15 sec, then temperature 60°C for time period 1 min, then temperature 95°C for time period15 sec and temperature 60°C for time period 15 sec.

Calculating Gene Expression Folding

we calculated the folding of gene expression for all genes in this research by using double ΔCT method (ΔΔCT) based on the following equation: [17].

Gene Expression folding = 2 ΔΔCT

DNA Extraction for methylation: DNA was isolated from the blood of all (60) samples included in the study using the DNA extraction kit supplied by Transgenbiotech company, then Nano drop device use to measurement the purity of extract DNA.

Methylation-Specific PCR in Promoters of the Per Family Gene

Period family (PER*1*, *PER2* and *PER3*) Genomic DNA methylation-specific PCR analysis was altered using sodium bisulfite, and methylation-specific PCRs were carried out largely in accordance with the instructions [18]. We examined the methylation status of the promoter regions of the Period family genes to detected whether irregular CpG methylation for the area of promoter was the cause for distinctive Period family gene expression in children with mental disorders. We created a pair of primers for methylation alleles in each of the PER genes. To detection the methylation sequence we used this Primer pairs :Per1-F ATTTAGGTTTACGTGCGTTC, Per1-R CGACTCAAAAACGAAAATCG, its annealing temperature was 60 and band size was 298 bp. Per2-F GCGGTTTCGTTGCGGTTTAC, Per2-R GCCGACGCCGTTTCAAACCG, its annealing temperature was 60 and band size was 140 bp. Per3-F CGGTTTTCGTTCGAGTTCGC, Per3-R ACGATTAATCGTCGAAACCG, its annealing temperature was 60 and band size was 145 bp [19]. After the Methylation-specific PCR program is done, PCR products were then loaded and electrophoresed on 2% agarose gels, stained with red safe deye and visualized under UV illumination [20].

Results and Discussion

Determined the Gene Expression Folding for PERs Family Genes

The studies result shown clear contrast in the level of gene expression for *PERs* family genes in the patients group when compared with control group as shown in the following tables (1, 2 and 3).

Table 1. Gene Expression for the PER1 Gene and the Housekeeping Gene

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. control	ΔΔCT	Folding
Control	27.14	22.71	4.43	4.43	0	1
Patients	30.72	24.39	6.32	4.43	1.89	0.33

Table 2. Gene Expression for the PER2 Gene and the Housekeeping Gene

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. control	ΔΔCT	Folding
Control	30.31	22.71	7.6	7.6	0	1
Patients	30.70	24.39	6.31	7.6	- 1.28	1.76

Table 3. Gene Expression for the PER3 Gene and the Housekeeping Gene

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. control	ΔΔCT	Folding
Control	31.71	22.71	9	9	0	1
Patients	32.35	24.39	7.96	9	- 1.04	1.58

Light and other environmental elements have a significant impact on the central pacemaker of SCN. Conversely, hormones and neural variables impact the circadian clock activity in peripheral tissues, which are then regulated by the central pacemaker through a variety of communication pathways [2]. The period family genes (*PER1*, *PER2*, and *PER3*) will regulation the central clock and peripheral tissue clock[18]. Many studies give an idea of that period family mutations was cause the disruption circadian clock, resulting in a shortened circadian period with decreased precision and stability[19].in our study , we found difference gene expression levels in the PER genes in kids have mental disorder, the gene expression folding of *PER1* (0.33) was decrease ,while the gene expression folding of *PER2*, *PER3* (1.76 , 1.58) was increase as compared with gene expression folding in healthy kids (1). Since expression of the period family genes act a major role in the circadian rhythm, this results proposed that the gene expression in circadian clock in the kids have mental disorder behaves differently from healthy kids.

Measuring the Gene Expression Level of NON-Coding RNA Regulate the PERs Family Genes

The studies result shown clear contrast in the level of gene expression for mi-RNA that regulate the *PERs* family genes in the patients compared within control group as shown in the following tables (4, 5 and 6).

Table 4. Gene Expression for mi-RNA24 that Regulate PER1 Gene and the Housekeeping Gene

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. control	ΔΔCT	Folding
Control	22.61	18.29	4.32	4.32	0	1
Patients	24.65	18.06	6.62	4.32	2.30	0.41

Table 5. Gene Expression for mi-RNA449a that Regulate PER2 Gene and the Housekeeping Gene

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. control	ΔΔCT	Folding
Control	25.59	18.76	6.83	6.83	0	1
Patients	28.24	19.11	9.13	6.83	2.30	0.20

Table 6. Gene Expression for mi-RNA103 that Regulate PER3 Gene and the Housekeeping Gene

No.	CT. target	CT. H.K	ΔCT. Target	ΔCT. control	ΔΔCT	Folding
Control	15.13	18.29	- 3.16	- 3.16	0	1
Patients	15.46	18.06	- 2.59	-3.16	0.59	0.56

We also found different levels of miRNA molecules that regulate period genes family in kids have mental disorder and healthy kids [21], the gene expression level of miRNA24, miRNA449a and miRNA103 that regulate *PER1, PER2* and *PER3* gene was decrease (0.41, 0.20, 0.56) as compared with gene expression folding in healthy kids (1). This is indicative of heterogeneity in regulation of period family genes in kids with mental disorder. This results may suggested a molecular basis for scheming clinical protocols for Early diagnosis for mental disorders depended on differential in the circadian clock between patient kids group and healthy group.

Results of Methylation-Specific PCR for the Per Family Genes

DNA methylation operation in the promoter of the *PERs* family gene, (50%) hyper-methylation in *PER1* gene, (43%) hyper-methylation *PER2* gene and (0%) no methylation in *PER3* gene, shown in the following figures (1, 2 and 3).

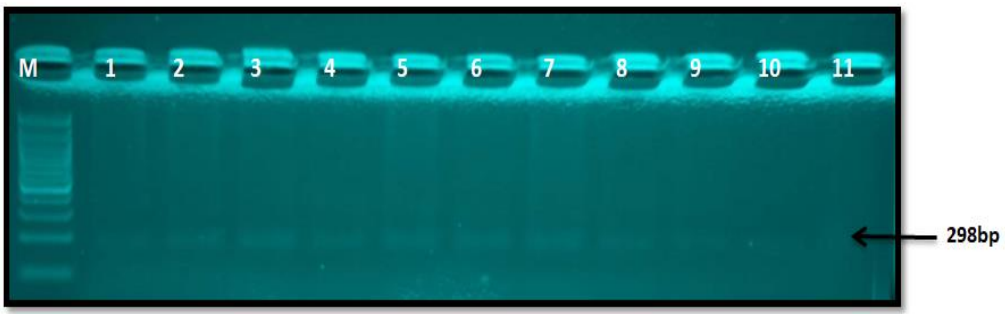

Figure 1. Results of Methylation-specific PCR reaction of Per1 gene

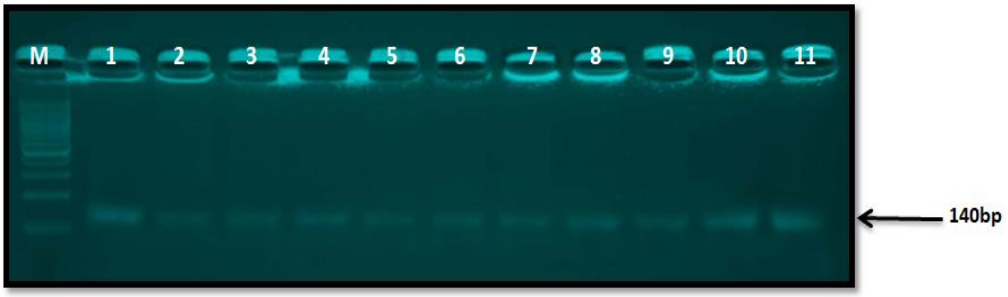

Figure 2. Results of Methylation-specific PCR reaction of Per2 gene

Figure 3. Results of Methylation-specific PCR reaction of Per3 gene

On the hand, this results be revealed the methylation of promoter sequences, considered as an epigenetic alteration, this will disable promoter functions cause dys-regulation and suppression the gene expression, we also find whether CpG methylation had taken place in the period family genes. In order to the *PER1* promoter was additional repeatedly methylated (50%) and *PER2* (43%), *PER1* and *PER2* was the focus of next studies. No connection between *PER3* methylation (0%) and mental disorders.

In our study We propose that the different expression of the PER proteins between patient kids group and healthy group is slightly due to promoter methylation of the *PER1* and *PER2* gene, resulting in dis-regulation of the PER proteins, or disordering of the signal transduction pathway or cell cycle impress the PER protein expression. In the methylation study of promoter *PER1*, we construct that 20 cases showed methylation in the *PER1* promoter and 17 cases showed methylation in promoter but no methylation was detected in the promoter in the PER3 gene. This difference in methylation resulting in disturbance of PERs gene expression and trouble in the circadian clock. On the other hand we proposed that period family genes inhibition act major importance role in the development of mental disorders.

Conclusion

The results of this study demonstrate increases in the gene expression folding of *PER2*, *PER3* and decrease in the gene expression folding of miRNA449a and miRNA103 that regulate *PER2* and *PER3* gene, while there is no clear difference between the gene expression folding of *PER1* and the gene expression folding of miRNA24, also show that the methylation status has a significant correlation with the genes expression folding of the period family genes and miRNA molecules that regulate it.

Acknowledgements

The authors are very grateful to the University of Mosul, Science College for the provision of their facilities, which helped to improve the quality of this work.

Reference

[1]. Parlak, G. C., Baris, I., Gul, S., Kavakli, I. H., 2023, Functional characterization of the CRY2 circadian clock component variant p. Ser420Phe revealed a new degradation pathway for CRY, *Journal of Biological Chemistry, JBC, 299*(12).

[2]. Barragán, R., Sorlí, J. V., Coltell, O., Gonzalez-Monje, I., Fernández-Carrión, R., Villamil, L. V., Asensio, E. M., 2022, Influence of DNA-polymorphisms in selected circadian clock genes on clock gene expression in subjects from the general population and their association with sleep duration, *Medicina*, *58*(9), 1294.

[3]. Gršković, P., Korać, P., 2022, Circadian gene variants in diseases, *Genes.*, *14*(9), 1703, 2023.

[4]. Alachkar, A., Lee, J., Asthana, K., Vakil Monfared, R., Chen, J., Alhassen, S., Baldi, P. ,2022, The hidden link between circadian entropy and mental health disorders, *Translational psychiatry*, 12(1), 281.

[5]. Baris, I., Ozcan, O., Kavakli, I. H., 2023, Single nucleotide polymorphisms (SNPs) in circadian genes: Impact on gene function and phenotype, *Advances in protein chemistry and structural biology*, 137-37.

[6]. Liu, C., Tang, X., Gong, Z., Zeng, W., Hou, Q., Lu, R., 2022, Circadian rhythm sleep disorders: genetics, mechanisms, and adverse effects on health, *Frontiers in Genetics*,13, 875342.

[7]. BaHammam, A. S., Pirzada, A., 2023, Timing matters: the interplay between early mealtime, circadian rhythms, gene expression, circadian hormones, and metabolism—a narrative review, *Clocks & Sleep*, *5*(3), 507-535.

[8]. Merrill, R. M., 2022, Mental health conditions according to stress and sleep disorders, *International journal of environmental research and public health*, *19*(13), 7957.

[9]. Pavithra, S., Aich, A., Chanda, A., Zohra, I. F., Gawade, P., Das, R. K.,2024, PER2 gene and its association with sleep-related disorders: A review, *Physiology & Behavior*,273:114411.

[10]. Kinoshita, C., Okamoto, Y., Aoyama, K., Nakaki, T., 2020, MicroRNA: a key player for the interplay of circadian rhythm abnormalities, sleep disorders and neurodegenerative diseases, *Clocks & sleep*,*2*(3), 282-307.

[11]. Du, N. H., Arpat, A. B., De Matos, M., Gatfield, D.,2014, MicroRNAs shape circadian hepatic gene expression on a transcriptome-wide scale, *eLife*, 3: e02510.

[12]. Na, Y. J., Sung, J. H., Lee, S. C., Lee, Y. J., Choi, Y. J., Park, W. H., Shin, H. S., Kim, J.H., 2005, Comprehensive analysis of microRNA-mRNA co-expression in circadian rhythm, *Exp. Mol. Med.*, 41:638–647.

[13]. Faltraco, F., Palm, D., Uzoni, A., Borchert, L., Simon, F., Tucha, O., Thome, J., 2021, Dopamine adjusts the circadian gene expression of Per2 and Per3 in human dermal fibroblasts from ADHD patients, *Journal of Neural Transmission*,128, 1135-1145.

[14]. Mosig, R. A., Kojima, S.,2022, Timing without coding: How do long non-coding RNAs regulate circadian rhythms?, *In Seminars in cell & developmental biology*,126, 79-86.

[15]. Dück, A., Reis, O., Wagner, H., Wunsch, K., Häßler, F., Kölch, M., Oster, H., 2022, Clock genes profiles as diagnostic tool in (childhood) ADHD—A pilot study, *Brain Sciences*, 12(9), 1198.

[16]. Kinoshita, C., Okamoto, Y., Aoyama, K., Nakaki, T.,2020, MicroRNA: a key player for the interplay of circadian rhythm abnormalities, sleep disorders and neurodegenerative diseases, *Clocks & sleep*, ***2***(3), 282-307.

[17]. Haimes, J., Kelley, M., 2013, Demonstration of a ΔΔCq Calculation Method to Compute Thermo Scientific Relative Gene Expression from qPCR Data, *Lafayette., CO: Thermo Scientific*.

[18]. Ramadan, Z., J., Hamed, O. M., Khalaf, I. H., 2020, Detection of genetic variation for some genes that related with recurrent spontaneous abortion in Nineveh province, *Biochemical & Cellular Archives*, 20(2).

[19]. Hameed, M. A., Hamed, O. M., 2023, Detection of P53 suppressor gene mutation in women with breast cancer in Mosul city, *AIP Conference Proceedings*, 2834(1), 020007.

[20]. Samblas, M., Milagro, F. I., Gómez-Abellán, P. J. A., Martínez, & Garaulet, M., 2016, Methylation on the circadian gene BMAL1 is associated with the effects of a weight loss intervention on serum lipid levels, *Journal of biological rhythms*, 31(3), 308-317.

[21]. Hamed, O. M., Al-Taii, R. A., Jankeer, M. H., 2021, Biochemical and genetic study in blood of β-thalassaemia children in mosul city, Iraq, *Iraqi Journal of Science*, 62(8), pp:2501–2508.

[22]. Salman, R. H., Mortatha, M. B., Nuiaa, R. R., 2024, Data Mining Technique for Diagnosing Autism Spectrum Disorder, *Iraqi Journal of Science*, 5239-5253.