University of Thi-Qar Journal Of Science (UTsci)

Website: jsci.utq.edu.iq **Email: jsci@utq.edu.iq**

Volume 7, Number 1, June 2019

diabetes mellitus patients in 2Assessment of melatonin level and genetic aspect of type Thi-Qar province, Iraq

Khalid G. Al-Fartosi Enas A. Jabbar Ahmed A. Jabbar*

Department of Biology - Collage of science - University of Thi-Qar, Iraq

***Email:-** Ahmedabdelhadijabbar@gmail.com

***Mobile:-** 07803106081

Abstract:-

The present study was designed to assessment of melatonin level and genetic aspects of patients with diabetes mellitus type 2 in Thi-Qar province, Iraq. The sample includes 80 patients compared with 40 as control group. The results showed a significant increase in levels of serum glucose in patients with DM type 2 compared with 40 as a control group at ($p<0.05$). The level of melatonin showed a significant decrease in patients of diabetes compared with the control group. The genetic study was conducted to identify the potential association of melatonin receptor *MTNR1B* gene SNPs with the progression of type 2diabetes mellitus (T2DM). In this same locus, four new SNPs were observed and their novelty were proven, including g.10718G>C, g.10863A>G, g.10865G>A, and g.10867T>A, while only one SNP was found to be known, namely rs1431442778. The current results indicated the presence of all of the observed SNPs only in the patients' samples.

Key words:- Diabetes type 2, Melatonin, Melatonin receptor, MTNR1B.

Introduction:-

Diabetes mellitus, or simply diabetes, is a group of diseases characterized by high blood glucose levels that result from defects in the body's ability to produce and/or use insulin. Several pathogenetic processes are involved in the development of diabetes. These include processes, which destroy the beta cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action (DeFronzo, 2009). The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (DeFronzo, 2009).

Melatonin is a pineal hormone under the control of the biological clock, which is located in the hypothalamus and regulated by period light exposure (Peschke and Mu¨hlbauer, 2010). Secretion of melatonin follows a diurnal pattern, typically peaking 3 to 5 hours when it is dark, (McMullan, 2013). Melatonin receptors have been found throughout the body in many tissues including pancreatic islet cells, reflecting the widespread effects of melatonin on physiological functions such as energy metabolism and the regulation of body weight (Claustrat *et al.*, 2005; Bonnefond *et al.*, 2012). Several lines of evidence suggest that melatonin may have a role in glucose metabolism (Picinato *et al.*, 2008). Ingestion of melatonin had a protective effect against the onset of diabetes in diabetes-prone rats with improvements also seen in the animals' cholesterol and triglycerides levels controls (Bonnefond *et al*., 2012).

In several large genome wide association studies, single nucleotide polymorphisms in the type B melatonin receptor (MTNR1B) were associated with higher levels of fasting glucose, higher levels of hemoglobin A1c, and increased incidence of gestational and type 2 diabetes. (Prunet-Marcassus *et al.*, 2003) Among these single nucleotide polymorphisms, those that cause loss of function of the melatonin receptor were associated with the highest incidence of type 2 diabetes.(Bonnefond A *et al*., 2012) .The effect of endogenous melatonin on glucose metabolism in humans is unknown, the animal data and human genetic studies suggest that either low melatonin secretion or

reduced melatonin signaling can impair insulin sensitivity and lead to type 2 diabetes.(Bouatia-Naji *et al*., 2009) A prospective association between melatonin secretion and type 2 diabetes , however, has not been reported. Thus, we performed a nested, case control study among women participating in the Nurses' Health Study to investigate the independent association of melatonin secretion and the incidence of type 2 diabetes (Prokopenko *et al.*, 2009 ; Soranzo *et al*., 2010).

Materials and methods:-

Study population:-

The target population of this study was 80 (40 males and 40 females) samples who are already diagnosed as diabetes patients by the consultant medical staff in AL- Nasiriya endocrine and diabetes center, Thi-Qar province, Iraq during the period from December 2017 to April 2018 with age ranged between (30 to 65 year) . A control group is composed of 40 healthy (20 males and 20 females) with the same age range.

Blood sample:-

Blood samples were obtained by venipuncture, from patients and control group. The blood sample was divided into two aliquots, which were, 1.5 and 3 ml. The first aliquot was dispensed in sterilized tubes with EDTA to prevent coagulation and this used to genetic detection of MTRN1B gene. The second aliquot was dispensed in a plain tube, and left for 15 minutes at room temperature to clot. Then, it was centrifuged at 3000 rpm for 10 minutes to collect serum and kept in the freezer (-20ºC) until use unless used immediately to analyze biochemical parameters.

Measurement of serum glucose:-

Serum glucose were analyzed by enzymatic colorimetric method by UV/VIS spectrophotometer, Japan using Kits supplied by Biolabo (France). (Allan and Dawson, 1979).

Melatonin assay:-

The melatonin levels were analysed in duplicate using commercially available Human MT (Melatonin) ELISA Kit, This ELISA kit applies to the in vitro quantitative determination of Human MT concentrations in serum and the minimum detectable dose of Human MT is 9.375pg/mL (The sensitivity of this assay, or lowest detectable limit was defined as the lowest protein concentration that could be differentiated from zero).

DNA extraction:-

DNA samples were extracted by using Genaid Kit according to manufacturer's instructions (using a mammalian genomic DNA extraction kit (Geneaid Biotech, Taiwan). The concentration and purity of DNA were measured by a nanodrop, while the DNA integrity was checked by a standard 0.8% (w/v) agarose gel electrophoresis that is pre-stained with a higher concentration of ethidium bromide (0.7 µg/ml) in TAE buffer, using a 1 kb ladder as a molecular weight marker. The isolated DNA was used as a template for PCR.

PCR:-

One PCR fragment was selected for amplification, which supposed to cover 390 bp of the *MTNRIB* gene that is positioned within the chromosome No. 11 in the *Homo sapiens* genomic sequences (table 1).

Table 1. The specific primers' pair selected to amplify 396 bp of SNP rs10830963 *MTNR1B* locus within the human genomic DNA sequences.

Primer	Sequence (5′-3′)	Amplicon size	Accession Number	Reference
MTNR1B-F	GCAGTCAGAAGCTGTGGTCTG ACA	396 bp*(1)	NG_028160.1 (10645–10668)	Vlassi *et al.* 2012
MTNR1B-R	GGCAAGGAACAGGGGCCACA		(11021–11040)	

*(1) The NCBI blastn engine has indicated that the actual length of the *MTNR1B* amplicon is 396 bp instead of 430 bp.

The lyophilized primers were purchased from Bioneer (Bioneer, Daejeon, South Korea). The PCR reaction was performed using *AccuPower* PCR premix. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The following program was applied in PCR. The amplification was begun by initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 95˚C for 15 sec, annealing at 57˚C for 20 sec, and elongation at 72˚C for 30 sec, and was finalized with a final extension at 72°C for 5 min (Vlassi *et al.* 2012). Amplification was verified by electrophoresis on an ethidium bromide (0.5 mg/ml) pre-stained 1.5% (w/v) agarose gel in 1× TBE buffer , using a 100-bp ladder as a molecular weight marker. It was made sure that all PCR resolved bands are specific and consisted of only

one clean and sharp band in order to be submitted into sequencing successfully.

Results and discussion:-

Glucose level:-

The result showed a significant increase (P≤0.05) in glucose levels in all DM patients compared with control group (table 2).

Table 2: Level of glucose in DM patients and control group

Parameters / Groups	Glucose level (mg\dl) Mean± SD
Control	101.62 ± 15.18 [b]
Patients	245.71 ± 82.83 [a]
L.S.D	20.53

-The different letters refers a significant different at P≤0.05

Type 2 diabetes is due to insufficient insulin production from beta cells in the setting of insulin resistance Insulin resistance, which is the inability of cells to respond adequately to normal levels of insulin, occurs primarily within the muscles, liver, and fat tissue(Farrell *et al.*, 2008). In the liver, insulin normally suppresses glucose release. However, in the setting of insulin resistance, the liver inappropriately releases glucose into the blood (Melmed *et al.*, 2011). The proportion of insulin resistance versus beta cell dysfunction differs among individuals, with some having primarily insulin resistance and only a minor defect in insulin secretion and others with slight insulin resistance and primarily a lack of insulin secretion (Masharani *et al.*, 2011).

Melatonin level:-

The result showed a significant decrease (P≤0.05) in melatonin level in all DM patients compared with the control group (table 3).

Table 3: Level of melatonin in DM patients and control group

Parameters / Groups	Melatonin (pg\ml) Mean± SD
Control	21.09 ± 3.68 [a]
Patients	19.27 ± 4.39 [b]
L.S.D	1.05

-The different letters refers a significant different at P≤0.05

Various studies have shown that melatonin may influence insulin secretion and glucose homeostasis. A low quantity of circulating melatonin occur in patients with type 2 diabetes (Prokopenko *et al.*, 2008), at the same time upregulated mRNA expression of melatonin membrane receptor was observed (Peschke *et al.*, 2007). Furthermore, polymorphisms in the melatonin receptor gene were linked with fasting blood glucose level and susceptibility to the occurrence of type 2diabetes (Rosen *et al.*, 2009). These clinical results indicate that melatonin improves glycemic control in blood and the insufficiency of melatonin might be associated with the development of type 2 diabetes. The investigated effects of melatonin on glucose homeostasis in young male Zucker diabetic fatty (ZDF) rats, an experimental model of metabolic syndrome and type 2 diabetes, showed that oral melatonin administration exert anti-hyperglycemic effect in young ZDF rats as insulin sensitizer and by improvement in β-cell function (Agil *et al.*, 2012). Polymerase chain reaction established that melatonin receptor deficiency have an effect on transcript levels of pancreatic islet hormones in addition to pancreatic and hepatic glucose transporters (Glut1 and 2) (Bazwinsky-Wutschke *et al.*, 2014).

The association between type 2 diabetes and some genetc variants within the *MTNR1B* gene was reported in several portions around the world (Valeriya *et al.*, 2009: Tuomi *et al.*, 2016). Therefore, the curent study was performed to assess such putative association regarding to Iraqi patients that suffering from the same type 2 diabetes conditions.

Sequencing of 396 bp amplicons of *MTNRIB* gene

Within this locus, twenty samples were included, which had shown exactly 396 bp amplicons length. The sequencing reactions indicated the exact positions after performing NCBI blastn for these PCR amplicons (Zhang *et al.*, 2000). Concerning the supposed 430 bp PCR amplicons of *MTNRIB* gene, NCBI BLASTn engine has shown extremely high sequences similarities between the sequenced samples and two specific targets, the first one was NG_028160.1 with about 99% of homology with the expected target that partially covered the *MTNRIB* gene that is positioned in chromosome number 11. By comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (GenBank acc. NG_028160.1), the exact positions and other details of the retrieved PCR fragments were identified (fig. 1). This PCR amplicon of 396 bp were found to occupy an

University of Thi-Qar Journal Of Science (UTsci)
Website: jsci.utq.edu.iq Email: jsci@utq.edu.iq
Volume 7, Number 1, June 2019

intronic region that positioned between exon1 and exon 2, respectively.

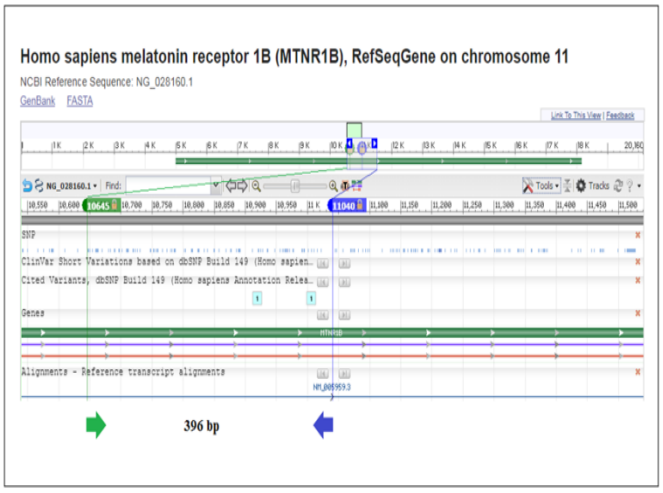
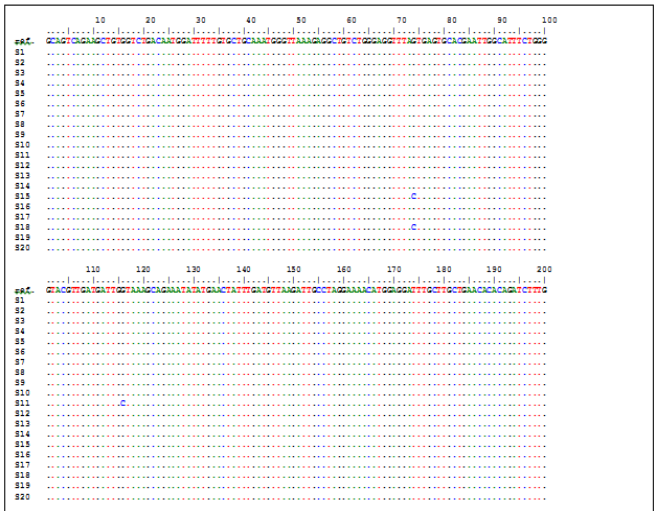


Fig. 1. The exact position of the retrieved 396 bp amplicon that partially covered the *MTNRIB* genetic sequences (acc no. NG_028160.1). The green arrow refers to the starting point of this amplicon while the cyan arrow refers to its end point.

After positioning the 396 bp amplicons' sequences within the *MTNRIB* DNA sequences, the details of its sequences were highlighted. Out of twenty analyzed samples, including 5 healthy control and 15 patient samples, respectively, the alignment results of the 396 bp samples revealed the presence of five mutations in the patients analyzed sample no. 11, 15, and 18 in comparison with the referring *MTNRIB* genetic sequences (fig. 2).



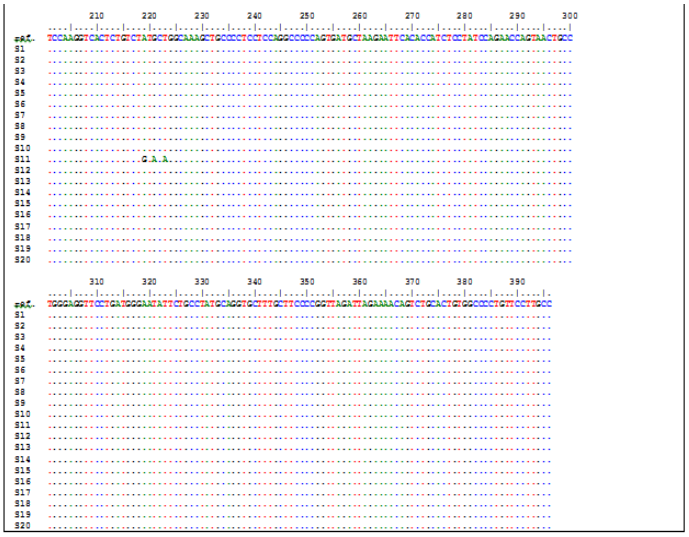


Fig. 2. DNA sequences alignment of twenty specimens with their corresponding reference sequences of the 396 bp amplicons of the *MTNRIB* genetic sequences. Each substitution mutation was highlighted according to its position in the PCR products. The symbol "ref" refers to the NCBI referring sequence. The letter "S" refers to the particular sample being analyzed, where S1 to S5 refer to healthy controls, while S6 to S20 refer to patients counterpart.

The sequencing chromatogram of each observed substitution mutation as well as its detailed annotations was documented as shown in (fig. 3).

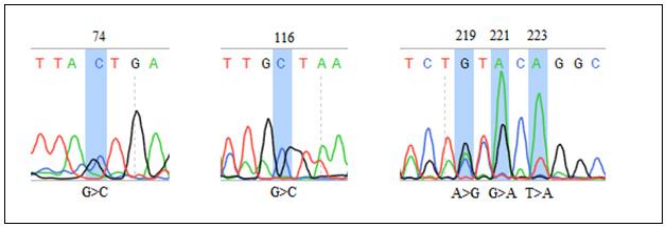


Fig. 3. The pattern of DNA chromatogram of the 396 bp amplicons of the *MTNRIB* genetic sequences. Each observed substitution mutation was highlighted according to its position in the PCR products. The symbol ">" refers to a substitution mutation.

SNPs characteristics check:-

To elucidate the positions of the observed SNPs with regard to their deposited SNP database of the sequenced 396 bp fragment, the corresponding positions of the *MTNRIB* gene were retrieved from dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/). To find out the nature of each observed SNP, a graphical representation was performed concerning the *MTNRIB*

dbSNP database within chromosome 11 (GenBank acc. No. NG_028160.1). The dbSNP engine has indicated that only one out of five observed SNPs, were previously known SNPs, namely rs1431442778 (Fig. 4). Whereas, the novelty of the other four discovered SNPs were validated, including g.10718G>C, g.10863A>G, g.10865G>A, and g.10867T>A. All five SNPs, whether being novel or known have found to cause only intronic variation between the first and the second exons of the *MTNR1B* gene.

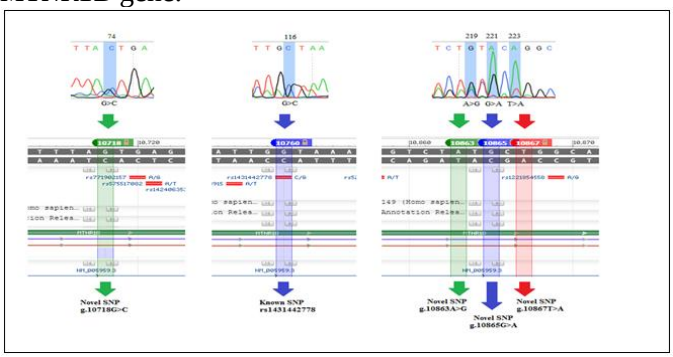


Fig.4. The *MTNRIB* PCR amplicons' SNPs novelty checking using dbSNP server. Each particular color refers to its corresponding SNP.

To sum up all the results obtained from the sequenced 396 bp fragments, the exact positions of the observed variations were mentioned in the NCBI reference sequences (table 4).

Table 4. The pattern of the observed SNPs in the identified *MTNRIB* sequences in comparison with the NCBI referring sequences of 396 bp amplicons (GenBank acc. no. NG_028160.1), in which, the annotation of all observed mutations were described.

No.	Mutation	Sample No.	position in the PCR amplicon	Position in the referring genome NG_028160.1	Type of SNP	SNP summary
1	G>C	S15 and S18	74	10718	Intronic variation	Novel SNP
2	G>C	S11	116	10760	Intronic variation	rs1431442778
3	A>G	S11	219	10863	Intronic variation	Novel SNP
4	G>A	S11	221	10865	Intronic variation	Novel SNP
5	T>A	S11	223	10867	Intronic variation	Novel SNP

The *MTNRIB* gene encodes one of two high-affinity forms of a receptor for melatonin, the primary hormone secreted by the pineal gland. This gene product is an integral membrane protein that is a G-protein coupled 7-transmembrane receptor (Pandi-Perumal *et al.*, 2008). It is found primarily in the retina and brain. It is thought to participate in light-dependent functions in the retina and may be involved in the neurobiological effects of melatonin. Since the production of melatonin diminishes the formation of cAMP in β-cells, the increasing level of *MTNR1B* gene expression on β-cells might damage insulin secretion due to diminished intracellular cyclic cAMP levels. Thus, the genetic polymorphisms in *MTNR1B* gene are a common genetic determinant of multiples insulin-related problems, such as increased fasting plasma glucose and risk for T2DM (Bouatia-Naji *et al.*, 2009).

The main limitation of the current research is correlated with the number of samples that only restricted by 15 patients. However, due to constraints in the samples collection restrict regulations and other problems, the number of samples have reduced the impact of this work in terms of samples number. Nevertheless, the current research has provided an obvious notion regarding the pattern of DNA polymorphism in the studied 396 bp fragment. This notion is originated from the concentrating of all the discovered mutations only in the patients' samples. The total discovered mutations in the present work are 5 mutations, namely g.10760G>C, g.10718G>C, g.10863A>G, g.10865G>A, and g.10867T>A. All of them were distributed only in the patients' samples, particularly in sample no. 11, 15, and 18. This observation indicates the pathological significance of the discovered mutations in the progression of T2DM. However, this disease-associated DNA polymorphism has not resulted from missense mutations as all the observed mutations were localized only in the intronic region that positioned between the first and second exon, respectively. These intronic variations of these observed SNPs have potentially participated in one or more metabolic alteration(s) that might trigger the development of T2DM. Therefore, it's not unusual for such mutations to participate in the development of this disease in Thi Qar population. Noteworthy, the present work has added four novel mutations in the *MTNR1B* genetic fragment, namely g.10718G>C, g.10863A>G, g.10865G>A, and g.10867T>A. This discovery may entail a special involvement of these four novel mutations in the progression of T2DM in the studied population. Therefore, it's highly recommended to broaden the current investigation to scan a large number of Thi Qar, as well as other locations that involved the southern parts of Iraq to discover the pattern of this novel polymorphic fragment association with the progression of T2DM.

References:-

Agil, A.; Rosado, I.; Ruiz, R.; *et al*. (2012). Melatonin improves glucose homeostasis in young Zucker diabetic fatty rats. *Journal of pineal research*, *52*(2), 203-210.

Bazwinsky- Wutschke, I.; Bieseke, L.; Mühlbauer, E.; *et al* . (2014). Influence of melatonin receptor signalling on parameters involved in blood glucose regulation. *Journal of pineal research*, *56*(1), 82-96.

Bonnefond, A.; Clément, N.; Fawcett, K.; *et al* . (2012). Rare MTNR1B variants impairing melatonin receptor 1B function contribute to type 2 diabetes. *Nature genetics*, *44*(3), 297

Bouatia-Naji, N.; Bonnefond, A.; Cavalcanti-Proença, C.; *et al*. (2009). A variant near MTNR1B is associated with increased fasting plasma glucose levels and type 2 diabetes risk. *Nature genetics*, *41*(1), 89

Claustrat, B.; Brun, J.; & Chazot, G. (2005). The basic physiology and pathophysiology of melatonin. *Sleep medicine reviews*, *9*(1), 11-24.

DeFronzo, R.A. "From the triumvirate to the „ominous octet": a new paradigm for the treatment of type 2 diabetes mellitus." *Clinical Diabetology* 10.3 (2009): 101-128.

Farrell, J. B.; Deshmukh, A. F.; & Baghaie, A. A. (2008). Low testosterone and the association with type 2 diabetes. *The Diabetes Educator*, *34*(5), 799-806.

Lyssenko, V.; Nagorny, C. L.; Erdos, M. R.; *et al* . (2009). Common variant in MTNR1B associated with increased risk of type 2 diabetes and impaired early insulin secretion. *Nature genetics*, *41*(1), 82.

Lyssenko, V.; Nagorny, C. L.; Erdos, M. R.; *et al*. (2009). Common variant in MTNR1B associated with increased risk of type 2 diabetes and impaired early insulin secretion. *Nature genetics*, *41*(1), 82

Masharani, U. E. & German, M. S. (2011). Pancreatic hormones and diabetes mellitus. *Greenspan's basic & clinical endocrinology*, *8*

McMullan, C. J.; Schernhammer, E. S.; Rimm, E. B.; *et al*. (2013). Melatonin secretion and the incidence of type 2 diabetes. *Journal of the American Medical Association*, *309*(13), 1388-1396.

Melmed, S. (2016). *Williams textbook of endocrinology*. Elsevier Health Sciences.

Pandi-Perumal, S. R.; Trakht, I. W.; Srinivasan, V. T.; *et al* . (2008). Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. *Progress in neurobiology*, *85*(3), 335-353.

Peschke, E.; and Eckhard M.; "New evidence for a role of melatonin in glucose regulation." *Best practice & research Clinical endocrinology & metabolism* 24.5 (2010): 829-841.

Peschke, E.; Stumpf, I.; Bazwinsky, I.; *et al*. (2007). Melatonin and type 2 diabetes–a possible link?. *Journal of pineal research*, *42*(4), 350-358

Picinato, M. C.; Hirata, A. E.; Cipolla- Neto, J.; Curi, R.; *et al* . (2008). Activation of insulin and IGF- 1 signaling pathways by melatonin through MT1 receptor in isolated rat pancreatic islets. *Journal of pineal research*, *44*(1), 88-94.

Prokopenko, I.; Langenberg, C.; Florez, J. C.; *et al* . (2009). Variants in MTNR1B influence fasting glucose levels. *Nature genetics*, *41*(1), 77.

Prunet-Marcassus, B.; Desbazeille, M.; Bros, A.; *et al*. (2003). Melatonin reduces body weight gain in Sprague Dawley rats with diet-induced obesity. *Endocrinology*, *144*(12), 5347-5352.

Rosen, R.; Hu, D. N.; Perez, V.; Tai, K.; *et al.* (2009). Urinary 6-sulfatoxymelatonin level in age-related macular degeneration patients. *Molecular vision*, *15*, 1673.

Soranzo, N.; Sanna, S.; Wheeler, E.; *et al.* (2010). Common variants at 10 genomic loci influence hemoglobin A1C levels via glycemic and nonglycemic pathways. *Diabetes*, *59*(12), 3229-3239

Tuomi, T.; Nagorny, C. L.; Singh, P.; *et al*. (2016). Increased melatonin signaling is a risk factor for type 2 diabetes. *Cell metabolism*, *23*(6), 1067-1077

Willi, C.; Bodenmann, P.; Ghali, W. A.; *et al.* (2007). Active smoking and the risk of type 2 diabetes: a systematic review and meta-analysis. *Journal of the American Medical Association*, *298*(22), 2654-2664

Zhang, Z.; Schwartz, S.; Wagner, L. *et al.* (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational biology*, *7*(1-2), 203-214