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# «Study of the expression of circulating microRNAs and Redox status markers as predictors of cardiovascular complications in Type II Diabetes Mellitus in patients over 40 years of age»

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## NORMATIVE REFERENCES

In this dissertation, references are made to the following standards:

GOST 7.32-2001 – Provisions on scientific research. Rules for structure and preparation.

GSSE RK 5.04.034-2011. Compulsory State Standard of Education of the Republic of Kazakhstan, postgraduate education, doctoral candidacy. Principal regulations (revised No. 1080 dated August 23, 2012);

GOST 7.1-2003 – Bibliographic record. Bibliographic description. General requirements and rules for structure.

## NOTATIONS AND ABBREVIATIONS

| | | |
|---|---|---|
| ACD | - | Acute cerebrovascular disease |
| ADIPOR1 | - | Adiponectin receptor 1 |
| ADA | - | American Diabetes Association |
| AGO | - | Argonaute |
| AUROC | - | Area under the receiver operating characteristics |
| AOPP | - | Advanced oxidation protein products |
| CAT | - | Catalasa |
| cDNA | | complementary DNA |
| CHF | - | Chronic heart failure |
| CVDs | - | Cardiovascular diseases |
| DFS | - | Diabetic Foot Syndrome |
| DGCR8 | - | DiGeorge Syndrome Critical Region 8 |
| DM | - | Diabetes mellitus |
| DNA | - | Deoxyribonucleic acid |
| EDTA | - | Ethylenediaminetetraacetic acid |
| ELISA | - | The enzyme-linked immunosorbent assay |
| EXPO-5 | - | Exportin 5 |
| FPG | - | Fasting plasma glucose |
| G-6-PD | - | Glucose-6-phosphate dehydrogenase |
| GPx | - | Glutathione peroxidase |
| GRd | - | Glutathione reductase |
| GSH | - | Glutathione |
| GSSG | - | Glutathione Persulfide |
| HIV | - | Human immunodeficiency viruses |
| HOMA-IR | - | Homeostatic Model Assessment for Insulin Resistance |
| IDF | - | International Diabetes Federation |
| IGT | - | Impaired Glucose Tolerance |
| IHD | - | Ischemic heart disease |
| IL-10 | - | Interleukin -10 |
| IL-18 | - | Interleukin -18 |
| IL-6 | - | Interleukin - 6 |
| IRS | - | Insulin Receptor Substrate |
| LPO | - | Lipid peroxidation |
| mRNA | - | Messenger ribonucleic acid |
| microRNA | | Micro ribonucleic acid |
| NADPH | - | Nicotinamide adenine dinucleotide phosphate |
| NF-κB | - | Nuclear factor kappa B |
| NO | - | Nitric oxide |
| NEM | - | N-Ethylmaleimide |
| OPA | - | Ortho-phthalaldehyde |
| PBS | - | Phosphate Buffer Saline |
| PCR | - | Polymerase chain reaction |
| RISC | - | RNA-induced silencing complex |

| | | |
|---|---|---|
| RNA | - | Ribonucleic acid |
| RNS | - | Reactive nitrogen species |
| ROC | - | Receiver operator characteristic |
| ROS | - | Reactive oxygen species |
| RT-qPCR | | A real-time quantitatively polymerase chain reaction |
| SOD | - | Superoxide dismutase |
| T1DM | - | Type 1 diabetes mellitus |
| T2DM | - | Type 2 diabetes mellitus |
| TNF-α | - | Tumor necrosis factor α |
| WEBSDR | - | The Wisconsin Epidemiologic Study of Diabetic Retinopathy |
| WHO | - | World Health Organization |

# INTRODUCTION

**General description of the research.** The dissertation is dedicated to a comprehensive study of microRNA expression, oxidative stress status, and inflammatory process and their role as biomarkers of cardiovascular complications in patients with Type 2 Diabetes Mellitus.

**Significance of the research.**

Type 2 diabetes mellitus is a complex metabolic disease prevalent worldwide. It is characterized by hyperglycemia resulting from tissue resistance to insulin. Patients often remain asymptomatic despite elevated blood glucose levels, complicating diagnosis. The clinical parameters used for diagnosis lack specificity, necessitating the identification of circulating markers for disease diagnosis and prognosis. Complications of type 2 diabetes include damage and dysfunction of vital organs such as the eyes (retinopathy), kidneys (nephropathies), nerves (neuropathies), and coronary vessels (cardiovascular disease).

The pathogenesis of T2DM is multifaceted, involving various pathophysiological and chronic inflammatory processes, including oxidative stress. Changes in the immune status of patients associated with impaired glucose metabolism can lead to vascular endothelial dysfunction. MicroRNA expression influences redox status and inflammatory processes, serving as biomarkers for cardiovascular complications. While numerous studies have investigated microRNA expression in diabetes, its role in vascular complications warrants further investigation.

In recent years, microRNAs (miRNAs), small non-coding RNAs approximately 18-22 nucleotides in length, have been extensively studied as crucial regulators of cellular functions. MicroRNAs participate in various pathological processes and have been detected and extracted from multiple biological fluids, including whole blood, serum, and plasma; they are very stable molecules easily detected in the bloodstream. Circulating microRNAs exhibit altered expression patterns depending on the physiological state, aiding in predicting, diagnosing, and monitoring metabolic diseases, including diabetes. Identifying and utilizing biomarkers involved in disease progression enables more accurate determination of exacerbation processes and facilitates the development of preventive strategies against vascular complications in T2DM.

It has now been established that oxidative stress is a component of metabolic disorders in diabetes mellitus and plays a crucial role in the development of macro- and microangiopathies in T2DM.

Oxidative stress has been established as a component of metabolic disorders in diabetes mellitus, playing a pivotal role in the development of macro- and microangiopathies in T2DM.

Increased oxidative metabolism in the cell contributes to the highest electron escape in the electron transport chain, increased redox potential, and generation of the superoxide radical ($O2^{-\bullet}$).

Numerous studies have convincingly demonstrated that chronic tissue inflammation is a crucial contributor to the development of T2DM. Inflammation is the immune system's initial response to tissue damage and is integral to the pathogenesis of various vascular diseases such as atherosclerosis and diabetic angiopathy. Consequently, cytokines, hormone-like protein substances produced by activated immune cells involved in intercellular communication, hematopoiesis, and inflammation, lack specificity for antigens and mediate immune responses and intersystem interactions.

Given the preceding, a comprehensive and systematic approach based on studying miRNA expression, redox status, and inflammatory processes as biomarkers of cardiovascular complications in patients with type 2 diabetes mellitus holds significant promise.

In connection with the previous, a comprehensive and systematic approach based on the study of microRNA expression, redox status, and inflammatory processes as biomarkers of cardiovascular complications in patients with type 2 diabetes mellitus is very promising.

**The purpose of the research is to study** microRNA expression, oxidative stress, and inflammation as new diagnostic markers for cardiovascular complications in patients with type 2 diabetes mellitus.

**To achieve the goal, the following tasks were set:**

1. Comparative analysis of biochemical parameters (HbA1c, glucose, insulin, HOMA-IR index, total cholesterol, triglycerides) in the blood of patients with type 2 diabetes mellitus.

2. To identify the relative expression levels of hsa-miR-21-5p and hsa-miR-126-5p in the blood plasma of patients with type 2 diabetes mellitus.

3. To assess the redox status by analyzing markers of oxidative damage in patients' blood and indicators of the endogenous antioxidant protection system.

4. To determine the spectrum of inflammatory markers (IL-6, IL-10, IL-18, and TNF-α) in the plasma of patients with type 2 diabetes mellitus and to analyze the correlation between inflammation markers, redox status, and relative microRNA expression levels.

5. To study the diagnostic value of markers associated with type 2 diabetes mellitus and its vascular complications in the study groups.

**The research objects and materials.** Peripheral blood samples from controls and DM2T patients with and without vascular complications.

**Research Methods.**

Anthropometric methods, lipid profile (enzymatic methods), the method of fluorescence spectroscopy (Bio-Tek Instruments Inc., Winooski, VT, USA), spectrophotometry method, UV-spectrophotometry method, enzyme-linked immunosorbent assay (ELISA), nucleic acid extraction, reverse transcription PCR, and real-time PCR methods. IBM SPSS Statistics carried out statistical analysis for MacOS, version 20.0; graphs were built using version 6 of GraphPad Prism.

**The scientific novelty of the research.**

The most significant scientific results of the dissertation are as follows:

- for the first time, multimap studies were conducted on biomaterial obtained from patients in a Mexican population diagnosed with type 2 diabetes mellitus, both without cardiovascular complications and with complications;

- circulating blood biochemical parameters (HbA1c, glucose, insulin, HOMA-IR index, total cholesterol, triglycerides) were investigated in patients with type 2 diabetes mellitus, both without and with vascular complications;

- the relative expression level of free circulating hsa-miR-21-5p, hsa-miR-126-5p as biomarkers at the epigenetic level was revealed;

- identified the redox status associated with oxidative stress in patients with type 2 diabetes mellitus, both without and with vascular complications;

- the concentration of inflammatory cytokines (IL-6, IL-18, IL-10, TNF-α) in the blood plasma of the studied groups of patients was determined;

The findings demonstrate the diagnostic value of specific markers associated with developing type 2 diabetes mellitus and its vascular complications in the studied groups.

**Theoretical significance of the research.**

This study's results significantly contribute to understanding the epigenetic mechanisms of type 2 diabetes, circulating miR-21 and miR-126, and biomarkers of inflammation and oxidative stress. Changes in microRNA expression play an important role in diagnosing complications of diabetes mellitus and in the development of micro- and macrovascular diseases.

Patients with diabetes engage in self-monitoring of blood glucose levels to reduce the risk of complications such as diabetic retinopathy, neuropathy, and atherosclerosis. However, despite achieving reasonable glycemic control, some patients still develop vascular complications. The mechanisms underlying metabolic memory are not fully understood, but it is hypothesized that elevated glucose levels may induce damage to cells surrounding blood vessels, further impairing their function. This phenomenon can result in a deterioration of microcirculation, an elevated risk of thrombosis, and the formation of blood clots, ultimately contributing to the development of vascular complications.

Modern molecular biomarkers used in clinical practice for the diagnosis of diabetes do not have sufficient sensitivity to predict the emergence and progress of complicated diseases. The study of epigenetic mechanisms, in particular, the expression of microRNAs, contributes to the expansion of the boundaries of our view and provides an opportunity to develop new methods of diagnostics and personalized means of treating patients. MicroRNA expression plays an essential role in the development of oxidative stress and chronic inflammation associated with diabetic complications. These new data are necessary for understanding the molecular mechanisms underlying the development of type 2 diabetes and its complications. They may provide the basis for developing effective ways to diagnose and prevent the disease.

**Practical significance of the research.**

The investigation of enzymatic and non-enzymatic antioxidant system factors allows a more profound comprehension of their impact on the efficacy of the body's

antioxidant defense against the development of vascular endothelial dysfunction. These findings will contribute to the effectiveness of preventive medicine in addressing type 2 diabetes mellitus development and the formulation of optimal protocols for managing and treating patients at risk of complications.

The results of the proposed research work were implemented in lectures and practical classes in the disciplines "Molecular endocrinology" and "Molecular membranology" for the curriculum of the educational program «7M05102-Biomedicine» of 2nd course of the Department of Biophysics, Biomedicine and Neuroscience, Faculty of Biology and Biotechnology, Kazakh National University named after Al-Farabi—act of introduction of completed research work into the educational process No.3 2023 – Application A.

**The main provisions for the defense:**

1. A difference was found in the comparative analysis of biochemical parameters (HbA1c, glucose, HOMA-IR index) in the blood of the control group, patients with T2DM without complications and with complications.

2. The relative level of microRNA expression as a prognostic biomarker in patients with T2DM without complications and with complications was analyzed.

3. The redox status of the control group differs from that of patients with T2DM without vascular complications and with complications.

4. The indicators of IL-6, IL-18, IL-10, and TNF-α associated with inflammation in blood plasma differed in the control group, in patients with T2DM without complications, and with complications, and the correlation of the results was analyzed.

5. It was proposed to consider hsa-miR-21-5p, SOD, LPO, and IL-6 as prognostic markers associated with the pathogenesis of T2DM and the occurrence of vascular complications.

The personal contribution of the dissertation student to the complication of the results of scientific work proposed for defense. All the results of the dissertation work were obtained in the presence and personal participation of the author. The dissertator independently analyzed the literature data on the topic of the study and experiments, processed and analyzed the study's results, and wrote and designed the dissertation manuscript.

**The levels of research organization**. The research described in this dissertation was performed on the physiological, biochemical, and epigenetic levels.

**Approbation of work.** The main provisions of the dissertation and research results were reported and discussed at international scientific conferences, “Kazakh National Medical University, named after S.D.Asfendiyarov” NJSC, RSE “Institute of Genetics and Physiology” of the Ministry of Education and Science of the Republic of Kazakhstan, NJSC “Kazakh National Women's Teacher Training University”.

**Connection of work with the scientific research program.**

The work was carried out within the framework of the international project of the Laboratory of «Comunicacion Intercellular» Grupo de Investigacion CTS-101 of the Center for Biomedical Research of the University of Granada (project leader - PhD, Professor Rusanova Iryna).

**Publications.** The majority of this dissertation content was published in 20 scientific works, including 4 articles in foreign journals (Antioxidants IF-7.675, Q1., Oxidative Medicine and Cellular Longevity, IF-6.543, Q2, Journal of Pharmacy and Nutrition Sciences Q3) including in Scopus and Web of Science databases; 3 articles in scientific journals recommended by Committee for the Provision of Science and Higher Education of the Ministry of Science and Higher Education of the Republic of Kazakhstan (CCESF MES RK); 13 theses were published in collections of materials of foreign and international-republican conferences.

**Dissertation structure.** This dissertation is 117 pages and contains notations and abbreviations, an introduction, a literature review, materials and methods, results and discussions, conclusions, references, and appendices from 236. Contains 11 tables and 30 figures.

# 1 LITERATURE REVIEW

## 1.1 Definition of diabetes and its classification

Diabetes mellitus (DM) is a disorder of insulin secretion and the effect of insulin on the body, a group of metabolic diseases characterized by chronic hyperglycemia resulting from two factors [1]. Since 1965, the World Health Organization (WHO) has periodically updated and revised the classification of diabetes mellitus (DM). In its first classification in 1965, WHO proposed the use of four age categories for diagnosis: infant or childhood (with onset between 0 and 14 years of age), young (onset between 15 and 24 years of age), and adult (onset age between 25 and 64 years of age) and elderly (with onset at age 65 years and older). In addition to the classification of diabetes by age, other types of diabetes have been proposed: juvenile, insulin-resistant, gestational, pancreatic, endocrine, and iatrogenic [2].

The most recent classification, still in use in most countries of the world, was proposed in 1999 and is considered the etiologic classification of diabetes. For the first time, the principle of dividing diabetes types according to the main etiopathogenetic mechanisms leading to the development of hyperglycemia was proposed. The need for insulin prescription no longer determined the type of diabetes. The 1999 classification abolished the concepts of "non-insulin-dependent" and "insulin-dependent" DM and proposed two main types - type 1 DM (T1DM) and type 2 DM (T2DM). It was emphasized that insulin therapy may be required for patients with either type of diabetes at some stage of the disease. In addition, the WHO recommended that not only the different aetiological types of diabetes but also the clinical stages of the disease be included in the classification [3]. The introduction of clinical stages indicated that people with diabetes, regardless of type, may progress through several stages, from normoglycemia to severe hyperglycemia with ketosis. T1DM is based on destroying β-cells, usually resulting in absolute insulin deficiency. In T2DM, two pathogenetic processes are observed: resistance of peripheral tissues to insulin action and impaired insulin secretion [4].

In 2019, WHO published a new classification of diabetes. Historically, clinical differences between the two main types of diabetes have been based on age of onset, degree of loss of β-cell function, degree of IR, presence of diabetes-associated autoantibodies, and need for life-saving insulin.

Type 1 diabetes and type 2 diabetes are heterogeneous diseases with significantly different clinical manifestations and course of the disease.

The classification of diabetes can be divided into the following general categories, each of which has its characteristics (Table 1):

**Table 1**- Classification of diabetes (WHO, 2019) [6]

| Types of DM | Definition |
|---|---|
| DM type 1 | Destruction of β-cells (mainly immune-mediated) and absolute deficiency insulin, beginning in childhood and early adulthood |
| DM type 2 | The most common type, different degrees of dysfunctional β-cell and IR, usually associated with |

| | |
|---|---|
| | excess weight and obesity |
| Hybrid forms: | |
| Slow developing immunomodulated DM adults | Similar to the slowly developing type 1 DM in adults, but often has metabolic symptoms syndrome, one type of autoantibody protects GAD with more extended function of β-cells |
| Type 2 diabetes is prone to ketosis. | Ketosis and insulin deficiency, but later, insulin is required; Frequent episodes of ketosis, or immune-mediated |
| Other specific types | |
| Monogenic DM (monogenic defect functions β-cell, monogenic insulin defect) | Caused by specific gene mutations, it has several clinical manifestations that require different treatments and occurs in childhood and adolescence. Caused by a particular mutation of the gene; has signs of severe IR without obesity; DM manifests itself when β-cells are no longer able to compensate for IR |
| DM as a result of diseases of the exocrine part of the pancreas | Associated with diseases and injuries of the exocrine part of the pancreas. |
| DM induced by drugs drugs or chemicals substances | It is induced by some drugs or chemical substances (nicotinic acid, glucocorticoids, thyroid hormones, α-Adreno mimetics, β-Adreno mimetics, thiazides, diazoxide, dilantin, pentamidine, pyriminil (Vacor), α-interferon). |
| Other genetic syndromes associated with DM | Many genetic disorders increase the risk of developing DM. |
| Infection in DM | It develops due to viral and bacterial infections (congenital rubella, cytomegalovirus, etc.). |
| Endocrinopathy | Associated with several endocrinopathies (acromegaly, hyperthyroidism, Cushing's syndrome, etc.) |
| Unusual specific forms of immunomodulated DM | Associated with rare immune diseases. |
| Unclassified DM | This category should be used temporarily when there is no clear diagnostic category in the debut of the disease. |
| Gestational diabetes | Diabetes diagnosed in the second or third trimester of pregnancy, without apparent diabetes before pregnancy. |

Type 1 diabetes is a polygenic multifactorial disease that leads to the development of absolute insulin deficiency, carbohydrate breakdown, and then the development of other types of metabolism [5]. This type of diabetes is most common in children and adolescents, but it can occur at any age. This is due to insufficient insulin production by the pancreas, and usually, insulin daily requires input.

Symptoms of the disease include constant thirst, increased urination, fatigue, and a significant decrease in body weight. The patient's condition worsens rapidly, and acetonuria develops. Clinical characteristics of these patients include a low body mass index, insulin prescription within 12 months of diagnosis, lack of timely insulin administration, and an increased incidence of ketoacidosis coma [7].

*Type 2 diabetes mellitus (T2DM)* is a complex metabolic disease characterized by hyperglycemia resulting from pancreatic β-cell dysfunction, insulin resistance, or decreased insulin sensitivity, leading to hyperinsulinemia to reduce elevated blood glucose levels. Gradually, due to the dysfunction of the islets of β cells of the pancreas, relatively insufficient production of insulin develops. T2DM is more common in the elderly over 40 years of age, although the prevalence of the disease in children and adolescents is constantly increasing due to increasing obesity rates. Symptoms include increased urination, thirst, fatigue, and slow healing of leg ulcers [8].

The 10$^{th}$ edition of the International Diabetes Federation Diabetes Atlas (IDF, 2021) estimates the prevalence of diabetes, impaired glucose tolerance, and the total number of people with diabetes will reach 643 million (1 in 9 adults) by 2030 and 784 million by 2045. (1 in 8 adults) and 10.2% of the world's population between the ages of 20 and 79 are estimated to suffer from type 2 diabetes [1]. 4 out of 5 people with diabetes (81%) live in low- and middle-income countries. Worldwide, 541 million adults, or 1 in 10, have impaired glucose tolerance, which increases their risk of developing type 2 diabetes [9].

Hybrid types:

*Adult-onset slowly developing immune-mediated DM.* The slow immune-mediated progression of adult DM is similar to the clinical manifestations of DM2T, and diet and the use of glucose-lowering drugs maintain normal carbohydrate metabolism during the first year of the disease. After that, however, decompensation of carbohydrate metabolism develops, requiring insulin therapy.

*Type 2 diabetes with a tendency to ketosis* is characterized by a cyclical course: an acute onset with severe hyperglycemia and ketoacidosis requiring insulin administration is followed by a period of remission during which insulin can be discontinued, and carbohydrate metabolism is restored by diet or hypoglycemic drugs. The duration of remission usually does not exceed 10 years [10].

*Other specific types of DM:*

*Monogenic DM (monogenic defects of β-cell function and insulin action).* Monogenic forms of DM do not exceed 5% of all DM cases, but the number of these patients is steadily increasing with molecular genetic studies' increasing possibilities and availability. Identification of monogenic types of DM not only determines the correct tactics of treatment but also predicts the course of the disease and the nature of its complications, explains the presence of other components of the syndrome in some cases, and also allows predicting the development of such disorders in close relatives [11].

*Diseases of the exocrine part of the pancreas.* Any process that causes diffuse damage to the pancreas can lead to DM. Such processes include pancreatitis, trauma, tumor, inflammation, infection, and pancreatectomy. Diabetes caused by pancreatic

disease (2.59 per 100,000 people) is more common than type 1 diabetes (1.64 per 100,000 people), but doctors often misclassify it as type 2 diabetes [12].

*DM is induced by drugs or chemicals. It occurs due to certain medications or chemicals that affect the pancreas and blood sugar levels. Depending on the causative factors, this condition can be temporary or long-term.*

*Other genetic syndromes associated with DM.* Many genetic disorders and chromosomal abnormalities increase the risk of developing diabetes [13].

*DM is caused by infection.* This is a type of diabetes that develops as a result of the influence of the infectious disease on the regulation of sugar levels in the body. Infection can cause temporary or permanent disruption of glucose metabolism, resulting in elevated blood sugar levels. When the body fights infection, various chemical signals and cytokines are released, which can affect the functioning of the cells responsible for regulating sugar levels and lead to insufficient production of insulin, the hormone responsible for glucose absorption by cells in the body. As a result, the blood sugar level increases [14].

*Endocrinopathy.* It occurs in diseases characterized by excessive secretion of hormones that are insulin antagonists. In addition, increased secretion of certain hormones such as cortisol, growth hormone, thyroid hormone, aldosterone, adrenaline, and gut hormones can also cause diabetes [15].

*Particular types of immune-mediated diabetes.* Some types of DM have a different pathogenesis or etiology than DM1T, and they are associated with certain immunologic diseases. Insulin receptor antibodies can cause diabetes by binding to insulin receptors and preventing insulin from binding to its receptors on specific tissues. In some cases, however, these antibodies can act as insulin agonists after binding to the receptor, causing hypoglycemia [16].

*Unclassified DM.* This category first appeared in the 2019 classification. The diagnosis "Unclassified DM" can be used temporarily, at the time of the onset of the disease, when it is complicated to determine the type of DM based on the clinical picture and additional studies or observations are necessary (determination of the level of C-peptide at the time of the onset of the disease or in the dynamics, antibody research, molecular genetic research). Experts emphasize that this is a temporary category, and the type of DM should be determined in the future.

*Gestational diabetes mellitus (GDM).* This type of diabetes develops in women during pregnancy and can lead to complications for both mother and baby. It usually disappears after the baby is born but may increase the risk of developing type 2 diabetes in the future [17]. In 2013, the WHO updated its recommendations for the classification of hyperglycemia occurring for the first time during pregnancy. Patients with GDM are a risk group for developing GDM in subsequent pregnancies, and patients with a high risk of developing DM should be under the supervision of an endocrinologist. In most cases of GDM, glucose regulation returns to normal after delivery. GDM occurs in approximately 7% of pregnant women (ranging from 1 to 14% in different populations), accounting for more than 200,000 cases annually [18].

In the future, the emergence of new knowledge about the etiopathogenetic mechanisms that cause the development of persistent hyperglycemia will undoubtedly lead to a revision of the classification of diabetes mellitus.

### 1.1.1 Dysfunction of the endothelium during diabetes

The endothelium is a single-cell layer that covers the surface of the space inside the vascular tubes and acts as a barrier between the blood and the vessel wall [19].

Endothelial cells perform many functions, including cell adhesion, tissue growth, and metabolism, angiogenesis, regulation of the inflammatory response, vascular integrity and permeability, hemostasis, vascular smooth muscle cell proliferation, fibrinolysis, thrombus formation and activation, and maintenance of blood flow. Disruption of this regulatory balance leads to endothelial dysfunction [20]. Endothelial cells are metabolically active and have paracrine, endocrine, and autocrine functions necessary to maintain vascular homeostasis under physiological conditions [21].

Currently, there are 4 typical forms of endothelial dysfunction — vasomotor, hemostatic, adhesive, and angiogenic. However, cases of isolated endothelial dysfunction are rare, and, as a rule, in most diseases, a combined violation of endothelial function is observed [22]. Endothelial dysfunction has been shown in animal models and macro- and microvascular complications caused by long-term, transient, and acute hyperglycemia in humans [23, 24]. In patients with diabetes, there is a decrease in the synthesis of vasodilators, which regulate the leading vascular tone, such as nitric oxide (NO) and prostacyclin, as well as an increase in the level of endothelin-1 and thromboxane A2 vasoconstrictors, which primarily indicate the vasomotor function of the endothelium [25]. In diabetes, there is an increase in the expression of molecular adhesion of the family of selectins and immunoglobulins, as well as molecular adhesion of platelets/endothelial cells (platelet/endothelial cell adhesion molecule 1, PECAM-1), thrombogenic biomarkers of endothelium - tissue factor (tissue factor, TF), inhibitor of activator plasminogen-1 (plasminogen activator inhibitor-1, PAI-1) Hyperglycemia is one of the factors in the alteration of the glycocalyx of endothelial cells, which is accompanied by a violation of the barrier function of the vascular wall, and an increase in its adhesive properties, in particular, due to the hyperexpression of vascular adhesion molecules on the surface of endotheliocytes (vascular cell adhesion molecule 1, VCAM-1), and in the absence of adequate glycemic control - P and E-selectins. [26].

It is worth noting the role of vascular endothelial growth factor (VEGF) in the occurrence and development of angiogenic endothelial dysfunction in diabetes, as it regulates the proliferation of vascular endothelial cells in various tissues. The adverse effects of increased VEGF synthesis are realized in a regulatory complex with other growth factors, including insulin-like, transforming growth factor beta, TGF-β, platelet, and others. Recent studies of disorders of angiogenic function in diabetes indicate that hyperglycemia directly induces the proliferation and differentiation of endothelial cells [27].

### 1.1.2 The role of hyperglycemia in the emergence of endothelial dysfunction

In the case of hyperglycemia, the polyol pathway of glucose oxidation is activated. With the help of aldose reductase, glucose is converted into osmotically active sorbitol and fructose, in this case, the synthesis of NO (nitric oxide, NOS) and the multimolecular enzyme complex nicotinamide adenine dinucleotide phosphate

(NADPH) bound to the cell membrane is of great importance in the functioning of the antioxidant systems of glutathione and vitamin E) is used [28]. NADPH deficiency activates the oxidation of free radicals and reduces NO synthesis, disrupting the antioxidant defense system [29]. In hyperglycemia, the diffusion rate of NO in primary smooth muscle cells slows down, the availability of L-arginine, a precursor of NO, decreases, the free radical degradation of NO, and the inactivation of other vasodilators increases [30].

The result of hyperglycemia-induced activation of the hexosamine pathway of glucose metabolism is a change in the activity of transcription factors such as specificity protein 1 (Sp1) and nuclear factor kappa-activated B cell light chain enhancer (NF-kB) [31].

In endothelial cells, the Sp1 protein induces the transcription of PAI-1, which reduces the thromboresistance of the vessel wall, and NF-kB promotes the production of pro-inflammatory cytokines [32].

An essential mechanism of the alternative effect of hyperglycemia is activating the diacylglycerol-protein kinase C signaling pathway. In Hyperglycemia during glycolysis, the concentration of dihydroxyacetone phosphate, an intermediate product in cells, increases; it is reduced to glycerol-3-phosphate and increases the synthesis of diacylglycerol, which activates protein kinase C [33]. Protein kinase C leads to the reconstruction of the structure of microcirculatory channels and causes an increase in the angiogenic activity of endotheliocytes. It also stimulates the production of VEGF, epidermal growth factor, and TGF-β. Activation of protein kinase C in smooth muscle cells through activation of NF-kB suppresses the activity of guanylate cyclase enzyme by inhibiting gene expression, with which NO exerts its effects, namely vasodilation [34]. In recent years, the literature has shown that glycemic control in patients with diabetes reduces changes in the blood vessel wall and prevents the development of clinical signs of angiopathy. There is evidence that adequate glycemic control in both type 1 and type 2 diabetes does not entirely block the pathogenetic mechanisms of the development of endothelial dysfunction but only slows down its development [35]. Therefore, hyperglycemia induces endothelial dysfunction in diabetes via multiple cellular mechanisms, including overactivation of protein kinase C (PKC) and the hexosamine pathway. Endothelial function plays an important role in maintaining the homeostasis of the body, and the currently formulated concept is that endothelial dysfunction is a central link in the pathogenesis of many diseases, including diabetes. The literature analysis shows that the development of endothelial dysfunction in diabetes consists of several vital aspects that can be presented in the form of a diagram (Figure 1).

Hyperglycemia, which causes the activation of polyol and hexosamine pathways, as well as the accumulation of glycolysis products in combination with the receptor and non-receptor effects of the end products of glycation, disrupt the metabolism and functioning of the signaling systems of endotheliocytes, both due to changes in the activity of enzymes, in particular, protein kinase C, endothelial NOS, and due to changes in the expression of several genes, including hyperexpression of NADPH-oxidase, PAI-1, VEGF, inducible NOS, and proinflammatory cytokines. The most significant manifestations of dysmetabolism of endotheliocytes in diabetes

should be considered oxidative stress and disturbances in the nitrogen oxide cycle.

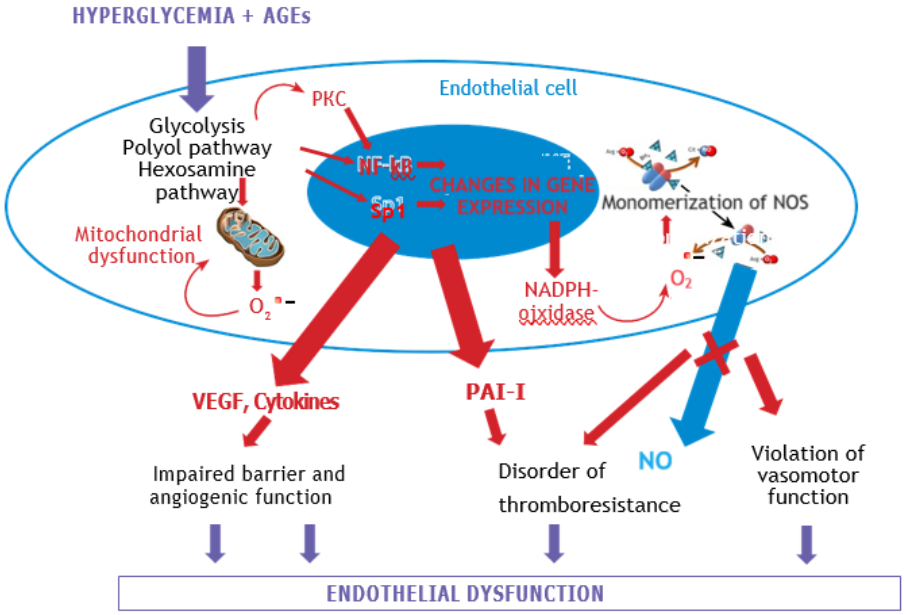



Figure 1- Scheme of the pathogenesis of endothelial dysfunction in diabetes [36]

Oxidative stress of endotheliocytes in diabetes mellitus is associated with the formation of pathogenic vicious circles, including mitochondrial dysfunction and monomerization of endothelial NOS, leading to persistent hyperproduction of reactive oxygen species, which in case of functional deficiency of antioxidant systems provides progressive damage and dysfunction of the endothelium.

Therefore, pathogenetic correction of endothelial dysfunction in diabetes mellitus should aim not only at ensuring adequate glycemic control but also at eliminating the phenomenon of oxidative stress. In this regard, a system of non-enzymatic and enzymatic antioxidants can act as a target for targeted therapy and prevention of angiopathy.

The result of oxidative stress of endothelial cells is the inhibition of endothelial NOS, a decrease in the production and bioavailability of NO, which causes a reduction in its ability to effectively exert paracrine dilatory effects on smooth muscle cells, causing impairment of vasomotor function, and, consequently, vascular tone and blood pressure. In addition, a decrease in the bioavailability of NO, including changes in paracrine interactions with platelets and leukocytes, as well as overexpression of PAI-1, impairs endothelial thromboresistance, which can cause intravascular microcirculation disorders, which inevitably lead to hypoxia, further aggravating endothelial dysfunction. Disruption of the autocrine regulatory effects of NO combined with overexpression of VEGF and proinflammatory cytokines causes changes in the barrier and angiogenic functions of endothelial cells, which causes structural remodeling of the vascular bed, leading to the development of angiopathy. Consequently, endothelial dysfunction in diabetes mellitus is manifested by a violation of all the main functions of the endothelium, including barrier, vasomotor,

angiogenic, and thromboresistance. Several effective therapeutic strategies currently used to treat patients with diabetic angiopathy are aimed at correcting individual functions of endothelial cells (treatment with vasodilators, antiplatelet agents, anticoagulants, and antiangiogenic therapy). To improve the effectiveness of such treatment regimens, further studies of the complex relationships between these disorders and the development of endothelial dysfunction in patients with diabetes are necessary [36]. Thus, endothelial dysfunction in DM is manifested by impairment of all primary endothelial functions, including barrier, vasomotor, angiogenic, and thromboresistance.

### 1.1.3 Micro- and macrovascular complications occurring in diabetes mellitus

The appearance of T2DM typically occurs 4–7 years before diagnosis; prolonged endothelial cell exposure to chronic hyperglycemia leads to endothelial dysfunction, considered one of the leading causes of T2DM complications [37, 38].

The following groups of chronic complications of diabetes mellitus are distinguished:

1. Diabetic angiopathy
– Microvascular complications (microangiopathies);
– Macrovascular complications (macroangiopathies);
2. Diabetic neuro osteoarthropathy.
3. Diabetic foot syndrome.

T2DM patients also have an increased risk for microvascular complications, including retinopathy, neuropathy, and nephropathy. Clinical studies show that treatments aiming only at glycemic control are ineffective in reducing vascular complications. New therapeutic targets are needed to mitigate vascular injury and prevent the development of vascular complications in people with diabetes.

Diabetic angiopathy is a generalized damage to blood vessels (mainly capillaries) in diabetes mellitus. It consists of damage to blood vessel walls and impaired hemostasis. Hormonal and metabolic disorders characteristic of diabetes are believed to play a leading role in the development of diabetic angiopathy [39].

In the pathogenesis of chronic complications of diabetes mellitus, the following factors are essential: polygenic inheritance, glucotoxicity (enzymatic glycation of proteins with the formation of advanced glycation end products, expression of growth factors, activation of cytokines, increased activity of protein kinase C, oxidative stress). etc.), dyslipidemia, hemodynamic disorders. [40].

*Diabetic neuropathy.* Diabetic neuropathy is a specific lesion of the peripheral nervous system caused by dysmetabolic processes in diabetes mellitus. It manifests itself as sensory disorders (paresthesia, numbness of the extremities), autonomic dysfunction (tachycardia, hypotension, dysphagia, diarrhea, anhidrosis), disorders of the genitourinary system, and other symptoms. According to WHO, diabetic polyneuropathy is a disease characterized by the progressive death of nerve fibers. The incidence of various forms of diabetic polyneuropathy reaches 65-80%. This complication accounts for 50-75% of lower limb amputations [41, 42].

*Diabetic retinopathy.* Diabetic retinopathy is an end-stage microvascular disorder and changes in the retina that lead to complete loss of vision. Diabetic retinopathy has the highest prevalence of blindness in patients with type 2 diabetes, accounting for more than 7-20% of cases at the onset of type 2 diabetes and 70-80% with a disease duration of more than 20 years. A feature of the development of diabetic retinopathy is that damage to the retinal vessels is not observed for a long time. In the early stages of the disease, there is no decrease in visual acuity. Only later, when the process moves to the retina's macular region, do complaints of blurred vision, distortion of objects, and changes in visual acuity appear [43-45].

*Diabetic nephropathy.* Diabetic nephropathy and chronic kidney disease are specific kidney damage in diabetes mellitus, accompanied by the formation of nodular glomerulosclerosis, which causes the development of end-stage renal failure requiring renal replacement therapy (dialysis, transplantation) [46]. The frequency of diabetic nephropathy depends on the duration of diabetes mellitus: with a duration of diabetes of 5 years - 7-10%, with a duration of 20-35 years - 20-35%, with a duration of the disease of more than 35 years - 50-57%. The main risk factors for diabetic nephropathy include chronic hyperglycemia, dyslipidemia, arterial hypertension, obesity, genetic predisposition, smoking, excess protein intake, etc. Diabetic nephropathy develops slowly, is asymptomatic for a long time, and is diagnosed through targeted studies. Diabetic nephropathy is one of the most severe and common complications in patients with diabetes, leading to end-stage renal failure with high morbidity and mortality. It is essential to identify patients prone to developing diabetic nephropathy to control the disease better. [47].

*Diabetic macroangiopathy* is a disease in which generalized atherosclerotic changes begin to develop in the arteries as a result of long-term diabetes mellitus.

Diabetic macroangiopathies include the following:

– Cardiac ischemia;

– Diabetic macroangiopathy of the leg veins;

– Cerebrovascular diseases.

Macrovascular complications, mainly cardiovascular and cerebrovascular diseases, continue to be the most common complications. In addition, diabetes is a leading cause of mortality and morbidity in patients with T2DM. Approximately 75% of these patients die from cardiovascular disease, including coronary artery disease, stroke, and peripheral arterial disease. [1].

According to the results of many studies, T2DM is a systemic cardiometabolic disease characterized by increased mortality from heart disease and stroke in men after 45 years of age and in women after 55 years of age, accounting for approximately 90% of all cases of diabetes [48, 49]. Myocardial infarction is asymptomatic in 1/4 of patients with diabetes. Due to the development of diabetes, the risk of cardiovascular death increases in men and 4 times in women. With all types of cardiovascular complications, myocardial infarction, stroke, angina pectoris, arrhythmia, and chronic heart failure, a high incidence of death is observed. The following positions are defined in the joint recommendations of the European Society of Cardiology and the European Association for the Study of Cardiovascular Diseases. There is a clear link between hyperglycemia and cardiovascular disease

(CVD). For every 1% increase in glycated hemoglobin (HbA1c), the risk of cardiovascular disease increases in some way. Compared to people without diabetes, the risk of CVD in men with diabetes is 2-3 times higher in women - 3-5 times. Postprandial glycemia is a more serious risk factor for cardiovascular disease than fasting glycemia [50].

*Coronary heart disease (CHD).* Type 2 diabetes mellitus is an independent risk factor for cardiovascular disease. The presence of diabetes increases the risk of developing coronary artery disease by 2-4 times. In the structure of mortality of patients with diabetes, the principal place is occupied by cardiovascular diseases, which are the cause of death in 52% of patients [51]. Both in the acute stage of myocardial infarction and during long-term follow-up, mortality in patients with diabetes is 1.5-2 times higher. At the time of diagnosis of type 2 diabetes, more than half of patients have coronary artery disease. The course of IHD depends on the duration of PD. Stable forms of coronary artery disease include angina pectoris, asymptomatic myocardial ischemia, myocardial infarction, chronic heart failure, arrhythmia, and acute coronary syndrome due to elevation or absence of ST-segment elevation [52].

*Acute coronary syndrome.* Acute coronary syndrome is one of the most common manifestations of coronary heart disease and is a group of clinical symptoms leading to myocardial infarction or unstable angina. Pathogenetically, the development of acute coronary syndrome is based on acute myocardial ischemia due to disturbances in coronary blood flow. The relationship between type 2 diabetes and acute coronary syndrome depends on atherosclerosis and inflammatory factors. People with diabetes have a high risk of developing atherosclerosis, characterized by the formation of "plaques" in the arteries. These "plaques" contribute to the blockage of blood vessels and the development of acute coronary artery disease. Mortality during the development of acute coronary syndrome is 2-3 times higher in patients with diabetes mellitus [53].

*Chronic heart failure (CHF).* HF is a clinical syndrome characterized by systolic, diastolic, or mixed myocardial dysfunction. Diabetes mellitus causes the development of heart failure regardless of the presence or absence of coronary artery disease due to the development of diabetic cardiomyopathy. The prevalence of heart failure in people with diabetes is 2-4 times higher than in people without diabetes. In the general population, the prevalence of heart failure is 1-4%, and 0.3-0.5% is due to type 2 diabetes mellitus along with heart failure. Studies of populations with CKD show a prevalence of type 2 diabetes of 12–30%. Successful treatment of patients with diabetes and its complications can significantly reduce the risk of developing CVD [54].

*Diabetic foot syndrome (DFS)* is a pathological condition in the form of complications of diabetes mellitus, purulent-necrotic processes of the lower foot, ulcers, and osteoarticular lesions developing against the background of changes in peripheral nerves, veins, skin, and soft tissues, bones, and joints. DFS is diagnosed in 4–10% of all patients with diabetes; the population of people with diabetes increases annually by 2.2–5.9%; 30–35% of patients belong to the high-risk group. The pathogenesis is based on distal neuropathy, microangiopathy, arthropathy,

osteoporosis, and disruption of the main blood flow in the veins of the lower extremities. Leg amputations in diabetic patients occur 17–45 times more often than in nondiabetic patients [55].

*Diabetes mellitus and arterial hypertension (AH).* Diabetes and hypertension always coexist. AH is one of the most dangerous factors in the development and progression of diabetic micro- and macroangiopathies. According to epidemiological studies, when diabetes and arterial hypertension are combined, the risk of developing fatal ischemic heart disease increases by 3-5 times, stroke by 3-4 times, complete loss of vision by 10-20 times, uremia by 20-25 times, and gangrene of the lower leg. – 20 times [56]. The frequency of AH in patients with diabetes is 2 times higher than the frequency of AH in the general population; it is 10-30% in patients with T1DM and 60-80% in T2DM. AH often precedes carbohydrate metabolism disorders. AH is detected at the onset of diabetes in 50% of patients with type 2 diabetes mellitus [57]. Chronic diabetes, caused by micro- and macrovascular damage, increases the risk of developing several other diseases and negatively affects the brain, kidneys, heart, and vision system.

### 1.2 History of microRNA discovery and general information

Thanks to molecular biology methods, new forms of the RNA family (tRNA, mRNA, rRNA) have been discovered recently. These include very short non-coding RNAs called ribonucleic acid (RNA) - "microRNA" or "miRNA." microRNAs are small, non-coding endogenous RNAs, with approximately 20–25 nucleotides that can modulate specific mRNA expression by pairing to the 30untranslated region (30UTR), causing inhibition of gene expression at the post-transcriptional level. Changes in circulating microRNA (c-miRNA) expression can help predict, diagnose, and monitor metabolic diseases, including diabetes [58, 59].

The first papers describing microRNAs were published in 1993 by teams led by V. Ambros and G. Ruvkun, who studied the mechanisms of developmental regulation in the nematode Caenorhabditis elegans. Still, their intensive study began in the early 2000s, following the discovery of let-7 microRNAs and the link between increased microRNA expression and malignant cell transformation [60, 61]. MiRNAs have been detected and extracted from different biological fluids, including whole blood, serum, and plasma, making them very stable molecules and easily detectable in circulation. Their potential use as biomarkers of human pathologies is possible due to their detection in 12 biological fluids [62].

These molecules have also been found to be involved in RNA interference, a process of repression of gene expression at the transcription, translation, deadenylation, or degradation of mRNA.

In 2006, Andrew Fire and Craig Mello were awarded the Nobel Prize in Physiology or Medicine for their 1998 work on RNA interference in the nematode Caenorhabditis elegans. In the following years, many articles were published describing the properties and structure of microRNAs, and many new microRNAs were discovered. Currently, 2693 mature microRNAs have been identified in humans [63].

To date, the following rules have been accepted for naming microRNAs and their predecessors:

1. First comes the three-letter designation of the species to whose genome the miRNA belongs. For example, "hsa" - Homo sapiens, "rno" - Rattus norvegicus, "mmu" - Mus musculus.

2. All microRNAs are named in the order and have the prefix “miR”. The first miRNAs discovered are named with the prefix “let”.

3. MicroRNA precursors (pre-microRNAs) are designated similarly, without the capital "R" in the prefix, "mir".

4. The sequence number of the microRNAs follows the prefixes.

5. The following are optional notations: if mature miRNAs have a similar sequence but differ by one or two nucleotides, the standard digital part of the name is followed by a letter ending. For example – hsa-miR-30a-5p и hsa-miR-30e-5p (uguaaacauccucgacuggaag и uguaaacauccuugacuggaag).

6. The suffix "-5p" or "-3p" indicates which pre-microRNA strand the mature molecule was transcribed from - sense or antisense. Previously, an "*" was used instead of the suffix to denote the "passenger" strand of the microRNA - the one that is expressed at much lower levels and is less likely to bind to the RISC complex. The “leading” miRNA strand did not have a suffix. For example – hsa-miR-30e – this is hsa-miR-30e- 5p, a hsa-miR-30e* - this is hsa-miR-30e-3p.

7. Pre-microRNAs, which process the same mature miRNAs but are located in different parts of the genome, have an additional numerical suffix in their names—for example, hsa-mir-194-1 and hsa-mir-194-2 [64]. To date, more than 2000 microRNAs have been identified as being involved in the regulation of approximately 1/3 of the human genome. This is due, on the one hand, to the dysregulation of cellular expression of microRNAs, which is observed in many pathologies characterized by increased or decreased expression, and, on the other hand, to the ability of the cell to produce or secrete a portion of microRNAs into the extracellular environment [65]. This suggests that they are present in biological fluids and that circulating microRNAs are useful as non-invasive biomarkers.

### 1.2.1 MicroRNAs in action: biogenesis, function and regulation

Before discussing the roles of miRNAs in the regulation and stress adaptation of cellular pathways, a brief review of miRNA biogenesis and mode of action is pertinent.

Many microRNAs are encoded by genes located in the introns of protein-coding genes. MicroRNA genes can also be located in exons, 5'-terminal and 3'-terminal untranslated regions of genes, and intergenic regions [66]. The biogenesis of microRNAs consists of several stages. The study's results show that the transcription of many microRNAs is regulated according to the principle of protein-coding genes.

MicroRNA undergoes a multi-step maturation process before it can affect protein translation. MicroRNA maturation pathways can be broadly divided into a basal or canonical maturation pathway, which occurs in most microRNAs, and an alternative or non-canonical pathway.

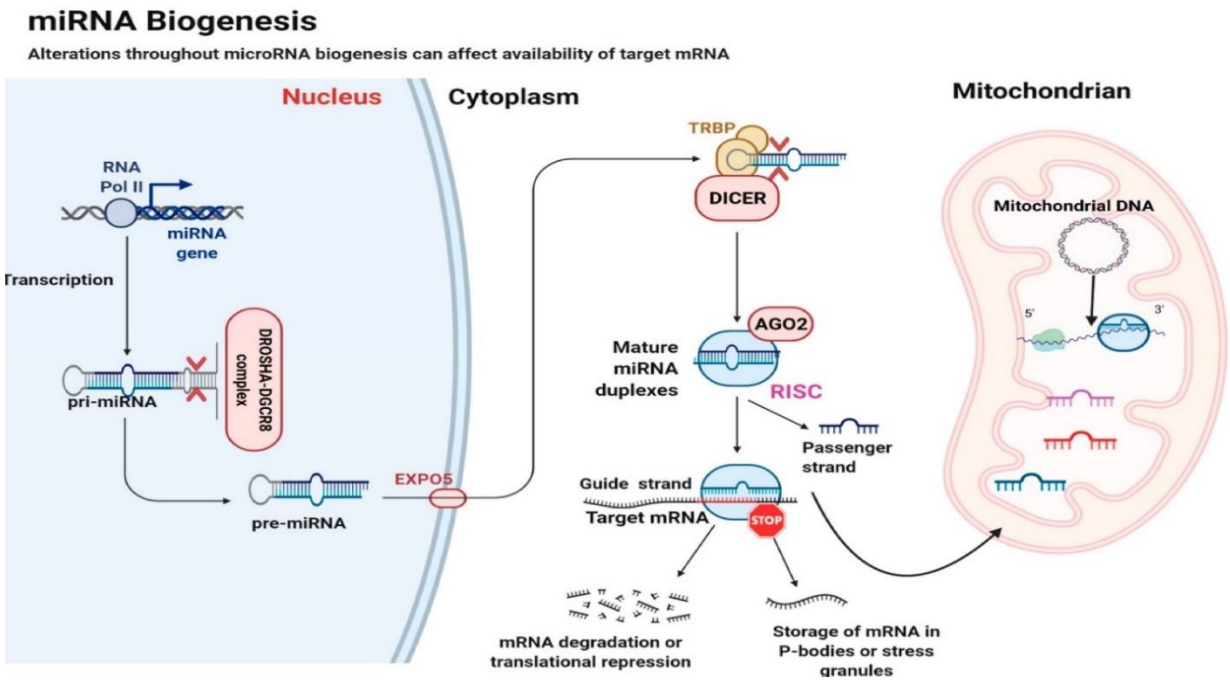



Figure 2 - MicroRNA biogenesis
(Image created with www.BioRender.com, accessed on 26 October 2021) [67]

The first is the traditional (canonical) pathway, which is carried out by forming a microRNA precursor molecule (pre-microRNA). Such a molecule has a characteristic morphology: "loops" with two single-stranded "tails" and several unpaired nucleotides in the central part. MicroRNA genes are transcribed in the nucleus as primary microRNA (pri-miRNA), a long transcript, predominantly by RNA polymerase II [68], less frequently by RNA polymerase III [69]. The next step is the specific recognition and excision of the pri-miRNA region by ribonuclease III, collectively called Drosha, and the RNA binding protein DGCR8, called Pasha - Partner of Drosha. The pre-miRNA is then exported to the cytosol via Exportin-5 (EXPO-5) [71], where another RNAse III endonuclease, called Dicer acts on the terminal loop of the hairpin structure of pre-miRNA to form a mature miRNA duplex. These miRNA duplexes are bound by Argonaute (AGO) proteins and other associated RNA-binding proteins, such as the transactivation response element RNA-binding protein (TRBP), to form the RNA-induced gene silencing complex (RISC). To associate the RISC complex with an appropriate mRNA transcript, only a single strand of mature miRNA that AGO-2 binds is needed, called the guide strand. The other miRNA strand (passenger strand) is rapidly degraded and removed. The 5′ region of the guide strand directs the whole RISC complex to mRNA complementarity at the 3′ UTR in a perfect or nearly perfect manner that ultimately leads either to complete degradation of the mRNA or sequestration of mRNA into cytosolic P-bodies or stress granules (resulting in translation suppression). Nuclear-encoded mature miRNAs have also been shown to enter mitochondria where these mitomiRs regulate multiple pathways. Together, these distinct characteristics are what make miRNAs major players in metabolic control and stress adaptation (Figure

2) [72, 73].

The degree of complementarity between the microRNA gene region, 6-8 nucleotides long, and the 3'-untranslated region of the mRNA object is often determined by the mechanism of regulation of gene expression. In turn, exosomes, complete with mRNA of the miRNA gene region, can be absorbed by recipient cells (including other cell types) in the cytoplasm of which the pre-microRNA is converted into mature miRNA. MicroRNAs can be released from the cell during apoptosis [74]. Circulating miRNAs change their expression depending on the body's physiological state and help predict, diagnose, and control metabolic diseases, including diabetes [75-77].

MicroRNA activity is characterized by extreme and pleiotropic activity. This means that multiple miRNAs can regulate the expression of one mRNA, and one miRNA binds to many mRNA targets, forming a complex regulatory system. As a result, a change in the expression of one miRNA leads to the shortening and degradation of the latter due to complementary binding of the expression of many mRNA targets.

In 2007, another way to make microRNAs was discovered. In this case, pre-miRNA is formed from short looping introns called myrtrons. The significance of the different ways microRNAs are formed remains to be studied. MicroRNAs function within individual cells and can enter the bloodstream and affect other cells in the animal's body. Most extracellular mature microRNAs (90-99%) are found in the blood as a complex with AGO (Argonaute) group proteins, catalytic components of RISC. It should be noted that microRNA gene expression, like protein-coding genes, is regulated at the epigenetic level during transcription, processing, and nuclear export and is also controlled by the extent of microRNA degradation. MicroRNA expression is a tissue-specific process, and the range of microRNAs used by an organism is directly related to the complexity of the organism's structure [78].

### 1.2.2. The role of microRNAs in the diagnosis and pathogenesis of type 2 diabetes

Many studies have shown that certain small single-stranded RNA molecules are associated with several diseases, such as diabetes and cardiovascular disease. Recent studies have identified the role of several diabetes-associated miRNAs in regulating key metabolic pathways in T2DM. Exposure to high concentrations of glucose reduces circulating miR-126 levels. miRNA-126 is widely distributed in vascular endothelial cells and is essential in the pathogenesis of diabetic complications of cerebrovascular diseases and diabetic nephropathy [79].

miRNA-146a levels have also been elevated in diabetic patients [80, 81]. Clinically, fluctuations in the circulating miR-26a and miR-146a levels may be predictors of different stages of T2DM [82]. MicroRNAs play a role in various biological processes, including adipocyte differentiation, metabolic integration, insulin resistance, and appetite regulation [83], and dysregulation of many microRNAs has been described in metabolic tissues of whole animals and humans [84, 86]. MicroRNA-221 is upregulated (increased) in adipose tissue in obese individuals and regulates a protein network associated with cellular metabolism,

especially the adiponectin receptor (ADIPOR1 - adiponectin receptor 1), which influences insulin sensitivity [85, 87]. MicroRNAs are also functionally involved in nephropathy [88, 89] and cardiovascular complications of T2DM [90, 91]. MicroRNAs can be quite stable in biological fluids, especially plasma and serum [92, 93]. Specific profiles of extracellular miRNAs in biological fluids and patterns of profile changes under disease conditions suggest their potential as valuable biomarkers for diagnosing and stratifying metabolic diseases [94, 95].

Using human plasma and serum samples to detect miRNAs may help detect the early onset of various diseases. To date, dozens of studies have characterized subsets of miRNAs. These results encourage further exploration of the potential clinical use of miRNAs as biomarkers of T2DM and complications.

Several studies indicate that most miRNAs (miR-144, miR-146a, miR-150, miR-182, miR-192, miR-29a, miR-30d, and miR-320) as candidate biomarkers of T2DM are involved in the regulation of insulin secretion, insulin resistance, glucose homeostasis or lipid metabolism involved in the pathology [96, 97]. At the same time, others are associated with the development of T2DM: miR-375, miR-96, miR-124a [98-100]. Previous studies have shown that a set of miRNAs, including miR-15a, miR-29b, miR-126, miR-223, and miR-28-3p, are dysregulated before developing T2DM.

In addition, other studies have shown that the profile of circulating microRNAs can be modulated by conditions of glucose homeostasis, correlating plasma microRNA levels with the degree of insulin resistance [101].

Overall, there is evidence that specific profiles of circulating microRNAs may be valuable biomarkers for identifying normoglycemic and diabetic individuals at high risk of developing T2DM. However, studies have not yet been conducted to test this hypothesis in a large population at high risk of developing T2DM.

Zampetaki et al. were the first to show that circulating microRNAs change their expression in 800 people with T2DM. Some miRNAs had low expression (miR-21, miR-24, miR-15a, miR-20b, miR-126, miR-191, miR-197, miR-223, miR-320, y miR-486), while miR-28-3p was overexpressed in plasma of T2DM patients compared to controls. In addition, using five miRs (miR-15a, miR-320, miR-126, miR-223 и miR-28-3p) can be identified in nearly 70% of patients with T2DM [102].

Since then, several studies have been conducted in different populations, some of which are summarized in the 2 tables below:

Table 2- MicroRNA expression in different populations

| Circulating microRNAs | Samples | Changes in expresión | n1- number of patients, n2- number of subjects in the control group. | Methods | References |
|---|---|---|---|---|---|
| miR-126, miR-423-5p, miR-192, miR- | plasma | increased | n1=35, n2=6 | quantitative polymerase | Ortega et al., 2014[103] |

| | | | | | |
|---|---|---|---|---|---|
| 195, miR-130b, miR-532-5p, miR-125b | | | | chain reaction (qPCR) | |
| miR-140-5p, miR-142-3p, miR-222 | | increased | | | |
| miR-126-3p | plasma | decreased | n1=193, n2=107 | RT-qPCR | Olivieri et al., 2015[104] |
| miR-21-5p | | increased | | | |
| miR-126-3p, miR-146a | plasma | increased | n1=31, n2=12 | quantitative polymerase chain reaction (qPCR) | Seyhan et al., 2016 [105] |
| miR-146a | blood serum | decreased | n1=56, n2=40 | quantitative polymerase chain reaction (qPCR) | Baldeón et al., 2016 [106] |
| miR-103, miR-28-3p, miR-29a, miR-9, miR-30a-5p, miR-150. | plasma | decreased | n1=102, n2=360 | RT-qPCR | Jiménez-Lucena et al., 2018 [107] |
| miR-30a-5p miR-150 | | increased | | | |
| hsa-miR-455-5p, hsa-miR-454-3p, hsa-miR-144-3p, hsa-miR-96-5p. | plasma | increased | n1=10 n2=5 | microchips | Yang et al., 2017[108] |
| hsa-miR-409-3p, hsa-miR-665, hsa-miR-766-3p. | | decreased | | | |

Several studies have shown that most miRNAs (miR-144, miR-146a, miR-150, miR-182NA, miR-192, miR-29a, miR-30d, and miR-320) are involved in insulin secretion, insulin resistance, tolerance to glucose as a candidate biomarker of T2DM shows that it is involved in the regulation of lipid metabolism involved in homeostasis or pathology [109,110]. The development of TS2T depends on the parameters miRNA-375, miRNA-96, and miRNA-124a [111-113]. Although most of these miRNAs have been analyzed in tissues, these biomarkers should be looked for at the circulatory level.

High glucose concentrations were found to be associated with decreased expression of miR-126 [102, 114] and increased expression of miR-221 and miR-146a in endothelial cells [115]. Under normal conditions, miR-126 is highly expressed in endothelial cells and contributes to maintaining vascular integrity and angiogenesis. [116]. In addition, miR-126 has been studied as an inhibitor of insulin receptor substrate (IRS), which is required for the insulin receptor to perform its physiological function and respond to the presence of insulin in adipose tissue [117]. Several studies indicate that decreased miR-126 expression appears to be associated with T2DM [118]. Expression of miR-126 in the plasma of DM2 patients and diabetic patients with cardiovascular disease revealed its low expression in both

groups of patients compared to healthy subjects [119]. In addition, an increase in miR-126 expression was observed in diabetic patients after 6 months of treatment (exercise and diet) [118]. On the other hand, a recent study suggests that exosomes isolated from mesenchymal cord cells and transfected with miR-126 can improve the inflammatory status in diabetic rats *in vivo* and cultured retinal cells *in vitro* and *in vivo* [120].

miRNA-126-3p modulates blood vessel regeneration and migration (mobilization) of hematopoietic stem and progenitor cells from the bone marrow into the bloodstream, so it has been classified as an "angiomicroRNA." This was one of the first microRNAs that was found to have decreased concentrations in the blood of patients with T2DM. It decreased in endothelial tissues of mice with diabetes and peripheral artery disease [121–123, 114].

miRNA-21 has anti-apoptotic effects through different mechanisms [124, 125]. Some diabetes studies show that miR-21 is overexpressed in plasma, suggesting that plasma may be a good candidate for controlling hyperglycemic damage [126]. Its increase in plasma corresponds to oxidative damage and acceleration of complications caused by increased blood glucose levels [127].

Some recent studies suggest that changes in the expression of several microRNAs, including miR-146a-5p, miR-16-2-3p, miR-126-5p, and miR-30d, in the plasma of patients with diabetes may serve as an indicator of microvascular complications associated with the disease.

### 1.3 Oxidative Stress and Reactive Oxygen Species

Reactive Oxygen Species Active oxygen species (ROS) and nitrogen species (NOS) are general terms to describe free radicals and other non-radical reactive forms known as oxidants. They contain electrons interacting with various organic substrates such as lipids, proteins, and deoxyribonucleic acid (DNA). Free radicals are known to play a dual role as harmful and beneficial species, as they can be detrimental and beneficial to living systems [128]. At low to moderate levels, free radicals (ROS and NOS) have beneficial effects such as protection against infectious agents, induction of mitogenic response, and maturation of cellular structures. ROS can include free radicals (superoxide anion $O_2^-$ and hydroxyl radical $OH^-$) and other non-radical reactive oxygen species (hydrogen peroxide $H_2O_2$) [129].

In addition, active nitrogen forms are also derivatives of molecular oxygen and can be classified as ionic (peroxynitrite $ONOO^-$) and non-ionic (nitric oxide $NO^-$) active oxygen forms. Free radicals are highly reactive particles with one or more unpaired electrons in the outer electron shell. Free radicals destroy or damage the structure of cell membranes to gain an extra or second electron from other molecules. Other forms of non-radical ROS do not have free electrons and, therefore, have weak oxidizing properties. For example, $H_2O_2$ has no free valence electrons, but the molecule has unshared electron pairs between oxygen and hydrogen atoms, so it can exhibit oxidizing properties, although weaker than $O_2$ [130].

The primary role in initiating peroxide reactions is played by reactive oxygen species (ROS) such as superoxide ($*O_2^-$), hydrogen peroxide ($H_2O_2$), hydroperoxyl ($*HRO_2^-$), hydroxyl radical (*OH), peroxyl radical ($*RO_2$), resulting in damage to the

lipid bilayer of membranes, interaction of highly reactive products with the amino groups of phospholipids, disruption of the transport function of membranes and the activity of membrane-bound enzymes [131, 132].

Chemical reactions that lead to the formation of free radicals include:

- $O_2 + 1e– \rightarrow *O_2^–$;
- $O_2 + 2e– + 2H+ \rightarrow H_2O_2$;
- $O_2 + 3e– + 3H+ \rightarrow *OH + H_2O_2$;
- $O_2 + 4e– + 4H+ \rightarrow 2H_2O_2$.

Among the substances formed at the initial stage of peroxidation of unsaturated fatty acids are diene conjugates; an increase in their composition was found in diabetic patients with microangiopathy compared to patients without microangiopathy. The formation of highly active and unstable hydroperoxides of saturated and unsaturated fatty acids is considered the intermediate stage of peroxidation.

Elevated levels of lipid hydroperoxides are often observed in patients with chronic non-communicable diseases, including diabetes mellitus [49].

*Oxidative status of proteins in diabetes.*

Proteins are important components of the body. The interaction of free oxygen radicals with proteins leads to various disorders, such as protein fragmentation, changes in the structure of protein molecules, and their functional activity. Oxidized proteins include increased levels of advanced oxidation protein products (AOPP) from oxidized albumin, fibrinogen, and lipoproteins. Elevated levels of AOPP indicate oxidative stress and inflammation. Several studies have shown an association between elevated AOPP levels and age-related diseases [133].

There is increasing evidence that AOPP is involved in the pathogenesis of many diseases, as well as in the development of their biochemical abnormalities and clinical complications. This paper discusses the possibilities of treating these diseases by reducing AOPP levels, preventing, or inhibiting their formation.

An increase in AOPP was found in a group of oxidized protein compounds containing dityrosine formed during oxidative stress in the plasma of patients with diabetes mellitus, arterial hypertension, and atherosclerosis [134].

*Lipid peroxidation in diabetes mellitus.*

Several studies have been conducted to evaluate ROS induced lipid peroxidation and antioxidant status in diabetes, but the results have been inconsistent. It is known that free radical oxidation of lipids is an integral part of vital processes, including electron transport by flavin elements, renewal of lipid composition of biomembranes, oxidative phosphorylation in mitochondria, mitogenesis, the transmission of nerve impulses, and others. Peroxidation reactions, which are constantly occurring with the corresponding activity of all lipid-dependent membrane-bound enzymes, are closely related to the renewal of the lipid composition of cell membranes.

Lipid oxidation occurs under the action of free radicals, including the initiation, elongation, branching, and rupture of oxidative chains [135].

Malonic dialdehyde is a highly active compound whose accumulation indicates

the severity of oxidative stress in the body. Determination of malonic dialdehyde levels in plasma or serum plays an important role in studying the pathogenesis of some diseases and pathological conditions. It can also be used as a marker for the effectiveness of antioxidant therapy [136].

Lipid peroxidation results in the formation of peroxidized compounds or lipid oxidation products (LPO). These include lipid hydroperoxides (LOOH) and aldehydes such as malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE). The significant product of lipid peroxidation is lipid hydroperoxides (LOOH).

### 1.3.1 Cellular antioxidant defense systems

The balance of redox potential in the body under normal and pathological conditions is ensured by the action of a multicomponent system of antioxidant enzymes and chemical substances with antioxidant properties. Antioxidative enzymes belong to the so-called dehydrogenases or oxidoreductases. All oxidoreductases are protein molecules that often form complex, spatiotemporal supramolecular complexes. However, despite the complexity of the organization of these structures, they all act as catalysts for the overall reaction of electron transfer from one molecule to another. In this case, the first molecule gives up electrons (donor) and is reduced, and the second one accepts electrons (acceptor) and is oxidized. The specificity of the action of oxidoreductases is determined first of all by the nature of the donor, which determines the nature of the catalytic center of the enzyme. Reduced antioxidant defenses are an important component in the development of oxidative stress [137].

Enzymatic and non-enzymatic antioxidant defense systems prevent the formation of new reactive oxygen species and prevent oxidative damage to cell membranes, nucleic acids, and other biological structures caused by reactive oxygen species. It has pronounced antioxidant activity, the most studied of which are superoxide dismutase, glutathione peroxidase, catalase, and other enzymes. Non-enzymatic sources of antioxidants include ascorbic acid, tocopherol, uric acid, and reduced glutathione [138].

Among the significant enzymes of antioxidant defense is superoxide dismutase (SOD), which catalyzes the reaction of dismutation of the superoxide radical into hydrogen peroxide and oxygen: $2O_2^{*-} + 2H+ \rightarrow H_2O_2 + O_2$. Superoxide dismutase is an enzyme that belongs to the class of oxidoreductases. SOD catalyzes the dismutation of oxygen. It prevents the conversion of the superoxide anion radical to the highly toxic hydroxyl radical. By preventing the accumulation of superoxide anion, SOD protects many cell structures from the damaging effects of superoxide.

In the human body, SOD is present in all organs and tissues, but its greatest amount and activity is found in erythrocytes within cells. The enzyme is present at low levels in the extracellular space.

Modern biochemical methods have shown that various isoforms of SOD have redox activity, including Cu/Zn dependent SOD, which is localized in the cytoplasm of cells and contains copper and zinc, $Mn^{2+}$ dependent SOD - the leading enzyme of the mitochondrial antioxidant system [49, p.10]. In case of insufficient activity of $Mn^{2+}$-dependent SOD, superoxide interacts with hydrogen peroxide to form a hydroxyl radical that causes lipid peroxidation and mitochondrial membrane damage:

$H_2O_2 + O_2– \rightarrow OH^- + OH + O_2$ [139].

Under the influence of SOD, the dismutation reaction produces hydrogen peroxide ($H_2O_2$), which is less reactive than $O_2$-•. $H_2O_2$ is then broken down into inert molecules of oxygen ($O_2$) and water (H2O) in a reaction catalyzed by catalase. Another way $H_2O_2$ breaks down into water depends on the level of GPx activity. In addition, GPx is involved in regenerating free fatty acids, nucleotides, nucleic acids, and proteins. In neutralizing free radicals, GPx glutathione is oxidized and then reduced by glutathione reductase in the presence of NADPH [139].

Typically, the antioxidant system acts as a buffer and neutralizes free radicals. In pathological conditions, when the formation of OBT increases several times, the antioxidant system is depleted, which leads to a violation of the redox balance of the body [140].

The cause of redox imbalance in the body in diabetes is prolonged hyperglycemia, which leads to depletion of antioxidant system resources, glycosylation and inactivation of antioxidant enzymes by reducing glutathione stores, and $O_2^-$ hyperproduction. As a result, the disturbed redox balance of the body leads to the activation of oxidative stress and its development, as well as the development of diabetic complications. When oxidative stress is activated, large amounts of ROS react uncontrollably with the body's proteins, lipids, DNA, and RNA. ROS hydroperoxides with proteins, which can alter the tertiary structure of proteins and even cause protein aggregation and denaturation. This leads to impaired enzymatic and regulatory activity of many processes. The direct harmful effect of ROS on DNA is characterized by the breaking of chains and their modification, the formation of DNA hydroperoxides, and damage to chromosomes [141].

*Glutathione antioxidant enzyme system.* Glutathione (GSH) is a tripeptide of L-γ-glutamyl-L-cysteinylglycine. It has a molecular weight of 307 g/mol. Most glutathione is in the reduced form (GSH), where the sulfhydryl group of the cysteine is not disulfide bonded to the second glutathione. A small percentage of glutathione is oxidized and exists as a dimer of two peptide elements linked by a disulfide bond between the cysteines in the two molecules. Oxidized glutathione (GSSG) indicates normal cellular status and oxidative stress. The reduced glutathione (GSH/GSSG) ratio decreases as some chemicals react with GSH to form compounds or increase GSSG levels [142].

Measurements of GSH and GSSG are helpful in experimental systems because changes in the GSH/GSSG ratio are associated with disease, aging, and cell signaling events [143].

The structure of glutathion is shown in figure 3.

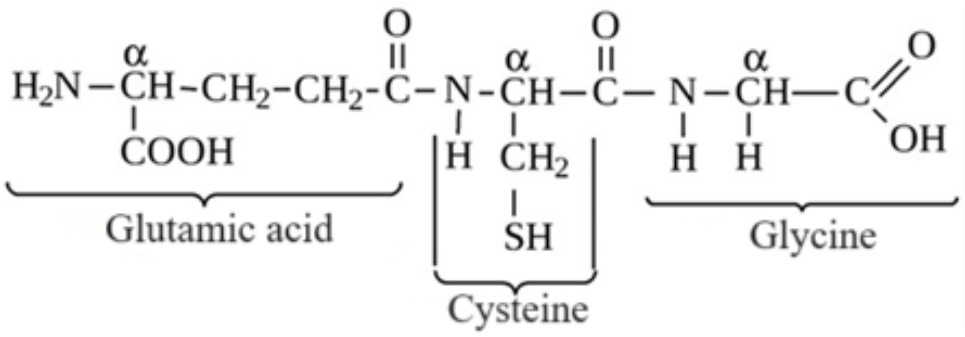



Figure 3 - Structure of glutathione [145]

The unique peptide γ-bond protects the tripeptide from destruction by aminopeptidases. GSH exists in several forms, including glutathione disulfide (GSSG), which is formed by oxidation, and a mixed form of glutathione, GSSR, which is cysteinyl glutathione disulfide.

Most GSH is found in the cytosol (80-85%) and, with lower concentrations, is found in the mitochondria and endoplasmic reticulum of eukaryotic cells. The GSH system is one of the major thiol-dependent antioxidant systems in mammalian cells. At the same time, GSH is the most abundant non-protein thiol in mammalian cells [144]. The glutathione synthesis rate decreases with age, and decreased GSH synthesis has been found in diseases such as diabetes, cystic fibrosis, and various liver diseases.

The decrease in GSH synthesis results from decreased expression of synthetic enzymes. In some cases, polymorphisms of different subunits of biosynthetic enzymes can reduce GSH synthesis by decreasing the activity of the synthetic enzymes. Since GSH is involved in removing reactive oxygen species from cells, a decrease in GSH production leads to an accumulation of reactive oxygen species in the cell [146]. Reactive oxygen species can damage DNA, proteins, and membranes, and reduced GSH expression has been implicated in atherosclerosis, HIV, some cancers, and rheumatoid arthritis.

### 1.3.2 The Role of Oxidative Stress in the Development of Vascular Complications in Diabetes Mellitus

A cell culture study using pancreatic β-cells, aortic smooth muscle, and endothelial cells demonstrated increased ROS production in diabetes [147]. Exposure of β-cell lines and islet cells isolated from the pancreas to oxidative stress has been shown to inhibit insulin gene promoter activity and mRNA expression, resulting in decreased insulin gene expression. Oxidative stress is also associated with chronic insulin resistance caused by hyperglycemia [148]. Experimental and clinical studies have shown that oxidative stress is involved in the pathogenesis of various diseases, such as cardiovascular diseases and carcinogenesis [149]. Chronic hyperglycemia is the main factor contributing to the development of micro- and macrovascular complications in T2DM. Hyperglycemia is responsible for DNA, lipid, and protein damage, the extent of which is related to the degree of hyperglycemia-induced ROS production and, consequently, oxidative stress [150]. Oxidative stress causes inactivation of proteins or enzymes such as SOD, GPx, catalase, and GSH, and the

downregulation of these proteins contributes to oxidative stress [151]. In diabetes, changes in the mitochondrial membrane lead to the activation of complexes in the electron transport chain, thereby promoting the formation of oxygen radicals. NADPH oxidase produces ROS and is considered the primary source of glucose-induced generation of reactive oxygen species in tissues and cells of diabetic models. It should be noted that xanthine oxidase plays an important role in the formation of ROS and in the development of diabetes and diabetic complications. During autoxidation in diabetes, glucose and its metabolites react with hydrogen peroxide in the presence of iron and copper ions to form hydroxyl radicals, thereby promoting the formation of ROS and the development of diabetic complications [152].

Oxidative stress, which causes pathological changes in human tissues and organs, plays an important role in developing micro- and macrovascular complications in T2DM [153].

Changes in glucose resistance, disruption of the 1st phase of insulin secretion, acute and chronic hyperglycemia, glucose toxicity, increased glucose autoxidation, and its participation in protein glycation processes cause the development of oxidative stress and stimulate the formation of free radicals that damage blood vessels. Walls and subsequently lead to endothelial dysfunction, causing disorders [154].

Numerous studies have shown that hyperglycemia, free fatty acids, and insulin resistance lead to the development of oxidative stress, vasoconstriction, inflammation, and thrombosis through activation of protein kinase C and advanced glycation end product receptors. An increase in blood glucose levels triggers a series of enzymatic cascades in mitochondria, including activation of NADPH oxidase, uncoupling of NO synthase, and stimulation of xanthine oxidase [155].

With insulin deficiency or increased insulin resistance, an imbalance occurs between pro-oxidants in the antioxidant defense system, as a result of the oxidation of carbohydrates and autoxidation of fatty acids, an increase in active oxidizing products, GPx, catalase, SOD, etc. decrease in the antioxidant defense system of enzymes, as well as non-enzymatic antioxidants (glutathione, vitamins K, E, C, etc.), impaired oxidative phosphorylation in mitochondria, changes in the biosynthesis of prostaglandins and leukotrienes under the influence of acute and chronic hyperglycemia, oxidation of mitochondrial proteins leading to mitochondrial dysfunction , and stimulation of the formation of reactive oxygen species, leading to oxidative stress, which are mechanisms for the formation of advanced glycation end products with activation of NADPH oxidase in endothelial and smooth muscle cells [156].

An imbalance between pro-oxidants and the antioxidant defense system is accompanied by increased processes of lipid peroxidation and a deterioration in the quality characteristics of lipoproteins, which contribute to the development of specific vascular changes involved in the development of atherosclerosis and endothelial dysfunction.

Currently, evidence has been obtained that hyperglycemia plays a direct role in the development of nitrosative stress when the formation of RNS exceeds the biological system's ability to neutralize and eliminate it [153 p.658]. Increased production of

free radicals, reactive metabolites, and oxidative stress are the main factors in the development of the disease.

### 1.4 The Role of Cytokines in Type 2 Diabetes

Cytokines are proteins produced predominantly by activated cells of the immune system. They do not have antigen specificity and mediate intercellular communications in the immune response, hematopoiesis, inflammation, and intersystem interactions.

Cytokines are traditionally divided into several groups: interleukins, which are factors of interaction between leukocytes; cytokines with antiviral activity – interferons; tumor necrosis factors; hematopoietic cytokines; chemokines are chemotactic cytokines [157].

Other research suggests that inflammation may be a key factor in the development of the disease, which has reached epidemic proportions worldwide. In connection with metabolic changes in diabetes, primarily at the cellular level, functional changes in the cell and changes in all its components in tissues with corresponding manifestations at the level of the inflammatory reaction should be expected [158].

Currently, 18 interleukins (ILs) are known and their main functions have been described. According to modern data, IL-1 plays an important role in the mechanisms of development of insulin deficiency. IL-1 is predominantly a waste product of macrophages, capable of stimulating a cascade of production of other cytokines (IL-2, 3, 4, 5, 6, etc.), causing activation of T and B lymphocytes. That is, a proinflammatory cytokine can induce a local inflammatory response and a systemic syndrome manifested by fever, hypotension and shock [159].

According to researchers, in order to correctly assess the immune status of the body, more is needed to study the cellular composition of the immune system, but also to assess the ability of these cells to produce cytokines. They regulate the differentiation of tissue cells of the red bone marrow, the maturation, reproduction and function of blood cells and the general immune system, the coordinated interaction of all parts of the immune system, and ensure the stability of internal homeostasis. Although they do not have antigen specificity, they are carriers of information about antigens and act as a specific cytokine complex. The role of the cytokine system in the development of pathological processes in the body is enormous. The main factor in increasing the synthesis of cytokines is the introduction of antigens into the body and the expression of cytokine genes, which cause an increase in cytokine levels in response to antigenic stimulation. The same cytokine can be produced by different types of body cells in each organ of the body [160].

On the other hand, cytokines have a wide opportunity to exchange biological actions with each other. Unlike other biologically active molecules, cytokines generally have autocrine, paracrine, and endocrine effects on cells. The role of cytokines in the formation of effector T and B lymphocytes and in the regulation of chronic inflammatory responses in pancreatic tissue is of interest to scientists studying diabetes. It is known that different subsets of T cells produce different sets

of cytokines. For example, the T helper cell type 1 (Th1) population secretes IL-2, IL-3 and INF-g, while the T helper cell type 2 (Th2) population secretes IL-4, IL-5, IL-10. and IL-13 [161].

Currently, scientists' opinions on the participation of various cytokines in the formation of diabetes are often contradictory, so this issue requires additional research. This is due to both pro-inflammatory and anti-inflammatory cytokines, the imbalance of which causes deviations in the protective properties of the immune response and negatively affects insulin secretion by pancreatic β-cells. All this requires continued scientific research into the role of the immune system in the pathogenesis and clinical features of diabetes, as well as the study of various development factors, as a particularly pressing problem in medicine [162].

Interferon (IFN) belongs to cytokines and is a group of proteins with antiviral, immunomodulatory and antitumor activity. The spectrum of the main biological effects of IFN suppresses the proliferation of intracellular infectious agents of viral and non-viral nature, slows down or enhances the synthesis of antibodies, stimulates macrophages, and enhances phagocytosis. In addition, it activates natural killer cells, enhances the cytotoxic effect of lymphocytes on certain cells, increases or inhibits the activity of a number of cellular enzymes, and causes changes in cell membranes [163].

Cytokines include the anti-inflammatory tumor necrosis factor (TNF), produced by cancer cells, macrophages, T and B lymphocytes and fibroblasts [164]. TNF is involved in the regulation of immune processes and apoptosis. Local exposure to TNF induces increased cell migration, activation of phagocytosis, increased production of proinflammatory cytokines, and a shift towards T helper cell involvement [165].

Obesity is a major risk factor for the development of T2DM. Chronic inflammation caused by obesity plays a key role in the pathogenesis of insulin resistance in T2DM [166]. Many inflammatory processes contribute to metabolic dysfunction [167].

Inflammatory cytokines such as IL-1, IL-6 and TNF-α have been confirmed to be involved in the development of insulin resistance. IL-8 levels have also been found to be elevated in patients with T2DM and associated with obesity-related indicators [168]. The anti-inflammatory system may reduce insulin resistance and diabetes sensitivity. It has been shown to exert protective effects against T2DM by inhibiting inflammatory cytokines [169,170].

Monoclonal antibodies that target inflammatory cytokines are used to treat coronary artery disease, rheumatoid arthritis, and other systemic autoimmune diseases.

# 2 RESEARCH MATERIALS ME METHODS

## 2.1 Research objects

In this work, patient selection and collection of biological material samples as research material was carried out on the basis of the Civil Hospital named after Dr. Juan I. Menchaca of Guadalajara (Guadalajara, Jalisco State, Mexico). The use of biological materials for research was conducted in accordance with the Declaration of Helsinki and Juan I. From the Ethics Committee of the New Civil Hospital of Menchaca (reference number: 17 CI 14 039 116 COFEPRIS - Federal Commission for the Protection of Sanitary Risks) and the Ethics Committee of the University of Granada ( reference number: 940/CEIH - Commission for Excellence and Innovation in Healthcare /2019) (Granada, Spain) was approved and performed according to international ethical requirements *(Appendix A).* Participation in this study, as well as biological samples, were collected only after the informed consent of the participants to participate in the study and the signing of the voluntary informed consent *(Appendix B).* The study was carried out with 47 men, 35 women, a total of 82 participants, classified into the three following groups:

Control (CG): Healthy volunteers with normal response to glucose and insulin, without a family history of first-degree diabetes, who did not have any endocrine, vascular, cardiac, or inflammatory pathologies, and was not taking any concomitant medication.

T2DM NC: Patients with controlled type 2 diabetes mellitus, without vascular complications and normal creatinine levels.

T2DM+C: Patients with type 2 diabetes mellitus that had micro- (diabetic retinopathy, nephropathy, and neuropathy) or macrovascular complications (stroke, coronary artery disease, and peripheral arterial insufficiency). All vascular events were documented in the patient's clinical file or clinical assessment.

The general criteria for inclusion for all groups comprised participants being over aged 40 to 65 years old and diagnosed with T2DM at least five years prior. Exclusion criteria included the existence of cancer, dialysis, amputations, pregnancy, inflammatory, infectious, or autoimmune diseases, and having a BMI greater than 40 $kg/m^2$.

The diagnosis of patients with T2DM was based on the criteria of the American Diabetes Association (ADA): fasting plasma glucose ≥ 126 mg/dL (7.0 mM), oral glucose tolerance test ≥ 200 mg/dL (11.1 mM), or glycosylated hemoglobin (HbA1c) ≥ 6.5%/48 mM [171].

## 2.2 Research materials

### 2.2.1 Collection of blood samples

All participants were advised not to take medication in the morning before blood sampling. After an overnight fast (10–12 h), fasting venous blood samples (5 mL) were collected in heparinized ethylenediaminetetraacetic acid (EDTA) tubes.

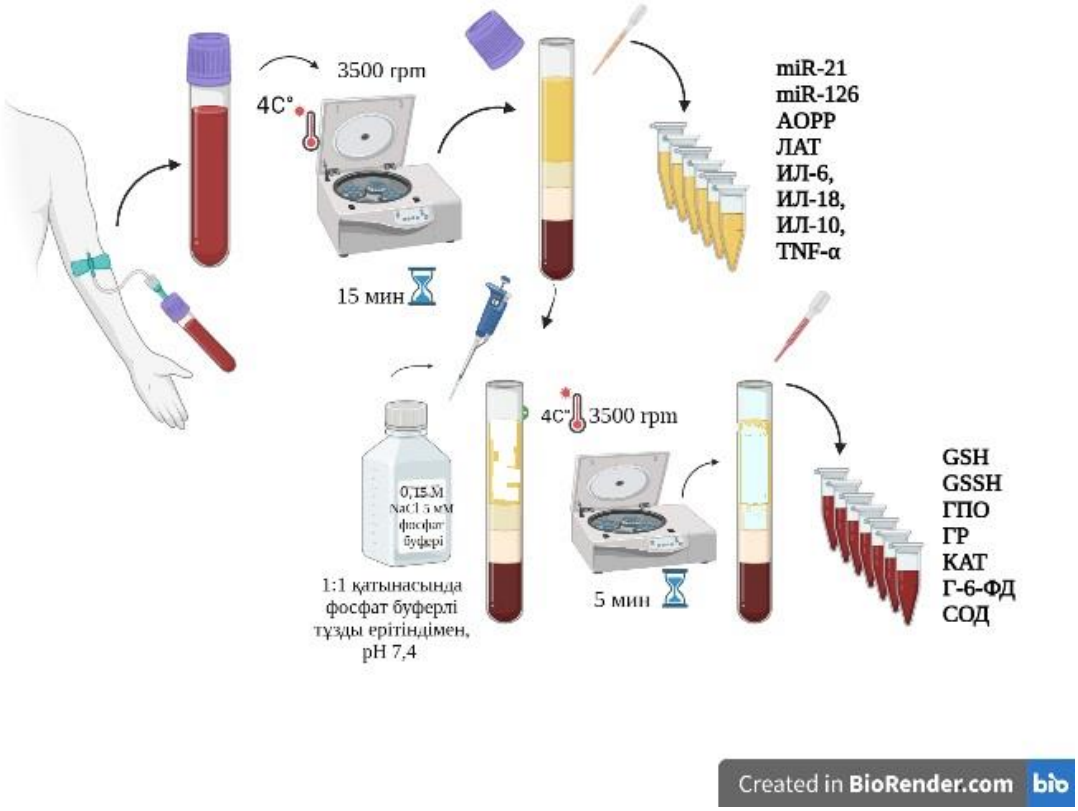



Figure 4 - Extraction of plasma and blood cells from a peripheral blood sample created by BioRender.com (June 04, 2023)

Peripheral blood from the participants was collected from the antecubital veins in tubes containing EDTA after a 12-h overnight fast. Samples were centrifuged at 3500 r/min for 15 min at 4°C. Plasma and erythrocytes were aliquoted in RNase/DNase-free tubes. The remaining erythrocytes in the test tube were suspended in a 1:1 ratio with phosphate-buffered saline, pH 7,4 (5 mM phosphate buffer containing 0,15 M NaCl) and centrifuged at 3500 rpm at 25°C for 5 min. Then, with a sterilized pipette, the physiological solution and leukocyte cells located on the top of the supernatant were removed, and the washed erythrocyte cells were separated and poured into test tubes. The prepared supernatants were labeled with the appropriate internal code and stored at -80°C (figure 4). Further processing of the biomaterial was performed at the "Intercellular Communication" Laboratory CTS-102 grupe of the Biomedical Research Center of the University of Granada Biomedical Research Center (Granada, Spain).

### 2.2.2 Chemical reagents

In chemical analysis, in conditions where a chemical reaction should occur while diluting the solution or adding other reagents to it, which does not change, and sulfides, hydroxides, carbonates, chromates, phosphates, etc. buffer solutions widely used during deposition: potassium phosphate buffer ( 50 mM potassium phosphate buffer pH=7.0, $KH_2 PO_4$ ); working buffer for catalase samples (working solution

working buffer 15 mM $H_2O_2$ ), phosphate - EDTA 10 mM, pH=6,25, EDTA-$Na_2$ 1 mM, buffer A or Hemolysis buffer EDTA 10 mM, pH=6,25, EDTA-$Na_2$ 1 mM; Buffer B: phosphate -EDTA, 100 mM, pH=,.0, EDTA-$Na_2$ 5 mM. $NaH_2PO_4 x2H_2O$ (PM=156,01 g/mol), $PO_4HNa_2$ (PM=142,0 g/mol); 50 mM potassium phosphate buffer pH 7.0 ($KH_2PO_4$) 6,8 g, hydrogen peroxide solution 15 mM $H_2O_2$; Ethylenediaminetetraacetic acid disodium salt EDTA-$Na_2$ (PM=372,2 g/mol).

Phosphate buffer 100 Mm, pH=7,5 ($PO_4H_2Na$ x $2H_2O$ PM=156,01 g/mol, $PO_4HNa_2$ PM=142,0 g/mol); Reduced glutathione GSH (PM=307,3 g/mol); Nicotinamide adenine dinucleotide phosphate NADPH (PM=833,3 g/mol): glutathione reductase enzyme (GSSG reductase); sodium azide ($N_3Na$,PM = 65,01 g/mol); Cumol hydroxide MW = 90,12 g/mol, = 0,83 g/ml);

GR Assay Buffer (10X); GR Sample Buffer (10X) - ; GR Glutathione Reductase ; GR GSSG; GR NADPH; Trichloroacetic acid 10% (TCA 10%); reduced glutathione (GSH, PM=307.3 g/mol); o-Phthalaldehyde (OPT, PM=134.1 g/mol); Standard curve for oxidized glutathione (GSSG, PM=612.6 g/mol); NaOH 0.1 N; N-ethylmaleimide (NEM, PM=125.1 g/mol):

Bicarbonate buffer 50 mM, EDTA-$Na_2$ 0.1 mM, pH=10.2 ($NaHCO_3$ 100mM, $Na_2CO_3$ 100mM); hydrochloric acid solution (1 M HCl); Ethanol (PM = 46.07); Chloroform (PM= 119.38);

Lipid peroxide solvent (acetonitrile, methanol); LPO reagent (N-methyl-2-phenylindole); LPO Reagent B ( Metianesulphonic acid); LPO standard ( 1,1,3,3-trimethoxypropane);

pH 7,4 in phosphate-buffered saline (PBS phosphate-buffered saline); chloramine-T (10 mmol/l) solution; Potassium iodide (KI, 1.16 mol/l); Acetic acid (96%);

Human Cytokine/Chemokine/Growth Factor Panel A Standard, Human Cytokine/Chemokine/Growth Factor Panel A Quality Controls 1 and 2, Human Cytokine Panel A Serum Matrix, Assay Buffer, 10X Wash Buffer, Human Cytokine/Chemokine/Growth Factor Panel A Detection Antibodies, Streptavidin-Phycoerythrin;

RPL Buffer, RPP Buffer, isopropanol, RWT Buffer, RPE Buffer, 80% ethanol, The poly(A) reaction mixture, TaqMan miRNA Reverse Transcription Kit, miR-Amp Primer Mix and TaqMan miR-Amp Master Mix, cDNA amplification mix consisting of primers, miR-Amp reaction products, miRNA TaqMan Advanced Assays Kit, TaqMan Fast Advanced Master Mix.

### 2.3 Research methods

#### 2.3.1 Clinical and anthropometric parameters

Clinical measures of anthropometry (age, sex, weight, BMI, waist circumference), systolic and diastolic blood pressure, and duration of illness at hospital admission or visit were determined using the standard anthropometric method. Body weight measurements were by standard weight scales. The BMI was calculated by dividing an individual's weight in kilograms by the square of height in meters:

$$BMI = \frac{m\ (kg)}{h\ (m^2)} \quad (1)$$

here, *m* - weight, kg;

*h*- height, $m^2$ ;

BMI - Body Mass Index kg/ $m^2$.

The waist circumference (WC) was measured using a standard tape measure. The WC was measured to the nearest 0.1 cm midpoint between the iliac crest and costal margin at the end of expiration [172]. Blood pressure was measured using a sphygmomanometer with an appropriately sized cuff after 5 minutes of rest. The average of two measurements taken 1 minute apart was used for systolic and diastolic blood pressure.

### 2.3.2 Determination of the biochemical parameters of the lipid profile and the carbohydrate metabolism

Biochemical parameters were analyzed in the clinical analysis service area of the New Civil Juan I. Hospital of Menchaca Guadalajara.

The following parameters were used: total cholesterol (TC) and triglyceride (TG) levels were assayed by colorimetric enzymatic methods.

Measurements Fasting plasma glucose (FPG) was detected by the Spein method using a Siemens analyzer (Siemens Healthcare, Erlangen, Germany), glycosylated hemoglobin (HbA1c) was measured by an automated analyzer (Tosoh, Japan), and insulin levels were detected by a Centaur XP automatic biochemical analyzer (Siemens, Siemens Healthcare, Erlangen, Germany). The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the equation:

$$HOMA - IR = \frac{\text{fasting plasma glucose (mg/dL)} \times \text{fasting insulin } \left(\frac{uU}{mL}\right)}{405} \quad (2)$$

Renal status was assessed by measuring the serum concentration of creatinine as an indicator of glomerular filtration rate and urea as an indicator of impaired kidney excretory function.

### 2.3.3 Determination of hemoglobin in red blood cells using the hemoglobin cyanide method (Drabkin method)

The principle of the hemoglobin cyanide method is based on the conversion of all types of hemoglobin into one hemoglobin cyanide.

The transformation of hemoglobin into hemoglobin cyanide was done by reaction with a transformation solution containing potassium ferricyanide, potassium cyanide, potassium dihydrogen phosphate and a nonionic detergent. Potassium dihydrogen phosphate maintains the pH level at which the reaction takes place within 3-5 minutes. Detergent enhances erythrocyte hemolysis and prevents cloudiness associated with plasma proteins [173].

According to the Drabkin method, 5 ml of Drabkin's solution was added to 20 µl of erythrocytes and incubated for 5 minutes at room temperature. Hemoglobin

oxidizes potassium ferrocyanide to form methemoglobin, which together with cyanide turns into potassium cyanmethemoglobin. The results were calculated according to the following formula:

$$\text{Hb}\left(\frac{\text{g}}{\text{l}}\right) = \text{Hemoglobin concentration in the sample} \times 37{,}7 \quad (3)$$

here, Hb – amount of hemoglobin, g/l;

37,7 – amount of hemoglobin in a standard solution, g/l.

Hemoglobin concentration of standard and sample was measured on BioTek *Power-Wavex Microplate Scanning plate* spectrophotometer, color intensity of cyanmethemoglobin at 540 nm wavelength is directly proportional to hemoglobin concentration in blood.

**2.3.4 Determination of the activity of catalase according to the Aebi method**

Determination of catalase activity was measured by the method of Aebi (1984) in a UV spectrophotometer with optical density at a wavelength of 240 nm for 3 minutes [174]. This method is based on the determination of the rate of decomposition of hydrogen peroxide by catalase of the studied sample by forming water and oxygen.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2H_2O+O_2$$

The principle of systematic determination of catalase activity consists of measuring the amount of hydrogen peroxide utilized by free catalases in a biological extract per unit of time. Catalase activity was calculated based on the amount of hydrogen peroxide utilized per minute in terms of specific activity.

As the catalase enzyme is located inside the red blood cells, it is diluted 1:20 in haemolysis buffer (phosphate-EDTA 10 mmol/L, pH=6,25, $Na_2$EDTA 1 mmol/L) and then centrifuged at 20000 g for 15 minutes, suspended. Then the prepared hemolysate was incubated in a ratio of 1:10, that is, 20 µl of the supernatant extract in an alkaline medium (50 mmol/l phosphate buffer, pH 7,0) in 180 µl of phosphate buffer. A diluted aliquot of 5 µl containing catalase was added to 495 µl working buffer containing phosphate buffer and hydrogen peroxide solution, and the rate of decomposition of hydrogen peroxide in the solution was determined.

The following formula was used to calculate catalase activity in µmol min per 1 mg of protein 1:

$$\text{Activity} = \frac{\Delta Absorbance\ /\ \text{min} \times 20000}{\varepsilon 240 * \text{conc Hb}\ (\text{g}\ /\ \text{l})} \quad (4)$$

here, the molar ratio of the absorption at a wavelength of ε240-240 nm is given;

Hb (g/l) is an indicator of hemoglobin;

ΔAbsorbance is the catalase value determined in a UV spectrophotometer;

20000 - dilution ratio of solutions.

Since the rate of dismutation of hydrogen peroxide in water and oxygen is proportional to the concentration of catalase, samples are first incubated with a known amount of hydrogen peroxide.

**2.3.5 Method for determining the activity of the enzyme superoxide dismutase**

Various isoforms of SOD catalyze the conversion of the superoxide anion radical to hydrogen peroxide and oxygen ( $O_2$•- +2H+ $\longrightarrow$ $H_2O_2$ + O2).

The determination of superoxide dismutase (SOD) activity in red blood cells is based on the ability of epinephrine (adrenaline) to inhibit the autooxidation reaction of adrenaline with the formation of superoxide anion in an alkaline environment at pH 10,2 [175].

$$RH_3^- + O_2^{\bullet -} + 2H^+ \xrightarrow{\text{SOD}} RH_3^{\bullet} + H_2O_2$$

here $RH_3^-$ is unoxidized epinephrine and $RH_3^{\bullet}$ is oxidized adrenaline (adrenochrome). Epinephrine is stable in an acidic environment but has a tendency to oxidize as the pH increases. Adrenochrome production is reduced in the presence of SOD because this enzyme neutralizes superoxide anion synthesis. The method was aimed at precisely determining Cu/Zn-SOD, therefore a chloroform-ethanol mixture was used to inactivate Mn-SOD and Fe-SOD. This is for the direct determination of Cu/Zn-SOD specificity. To evaluate the action of SOD, the 50% inhibitory dose (ID50) of hemolysate was calculated, that is, the amount of proteins capable of inhibiting the production of adrenochrome by 50%.

SOD was measured by a special spectrophotometric method. Erythrocyte fractions were diluted in distilled water ($H_2O$) at a ratio of 1:5 and hemolyzed. The ethanol-chloroform solution was added to the hemolyzed solution, shaken by hand for 60 seconds, and then centrifuged at 2200 g for 5 minutes to separate the SOD extract. After extraction of SOD, 6 mM adrenaline was prepared in 1 HCI protected from light to prevent its autooxidation. The separated aliquot was diluted in distilled water ($H_2O$) in the ratio of 1:25, 1:50, 3:50, and 2:25. Spectrophotometer 96 well plates were poured with a 5-fold dilution of the sample supernatant in order of decreasing SOD concentration. Then water, epinephrine (adrenaline) and sodium bicarbonate buffer (carbonate/bicarbonate buffer 50 mM, EDTA-$Na_2$ 0,1 mM, pH=10,2) are used in an alkaline environment for oxidizing adrenaline. The reaction of autoxidation of adrenaline into adrenochrome in the absence and presence of SOD in different quantities of the analyzed sample, all measurements are carried out by measuring the optical density of adrenochrome at 480 nm for 10 minutes on a *BioTek Power-Wavex Microplate Scanning* plate spectrophotometer. The activity of SOD was expressed as U/mg of haemoglobin, 1 unit is defined as the amount of enzyme that can inhibit adrenochrome production by 50% (Hb (1 U = 50% inhibition of adrenaline autoxidation).

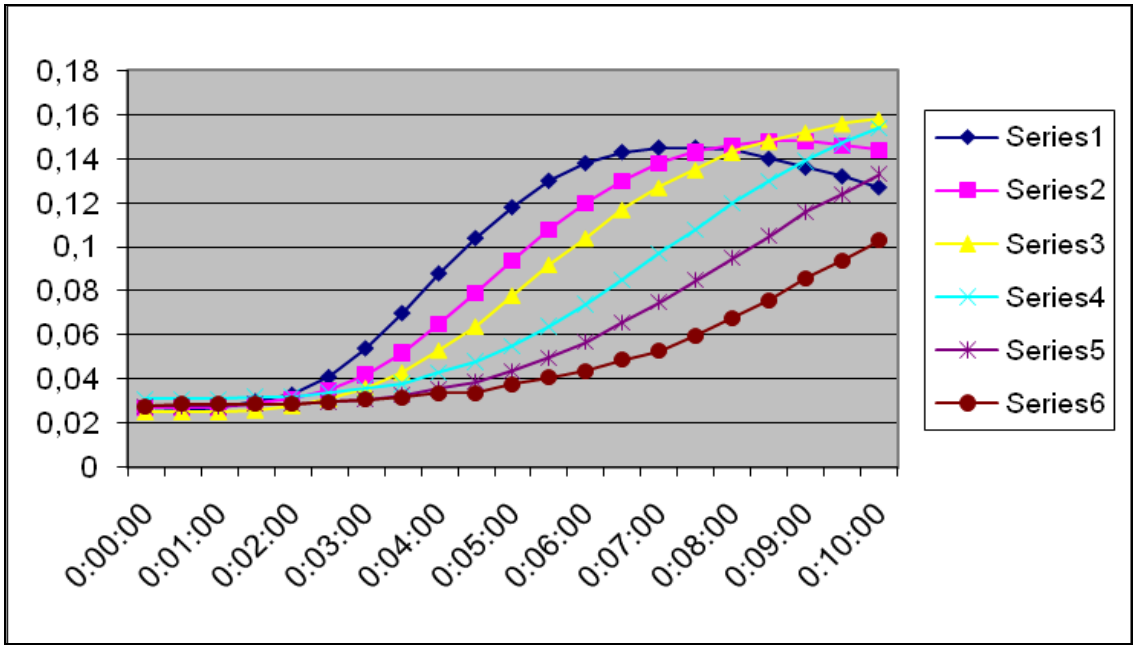



Figure 5 - Linear and logarithmic functions of adrenaline inhibition values at different levels of SOD

Calculations are made by finding the absorbance values in the time range, where the graph has a maximum slope and is approximately linear (Figure 5). The ratio of the difference of the presented absorbances between the time range is calculated. The linear and logarithmic functions of the values of inhibition of the amount of epinephrine in the presence of different concentrations of SOD are plotted on the spreadsheet. The function with the best fit (R2 closest to unity) is chosen. The SOD activity is expressed in relation to the hemoglobin content in the sample.

**2.3.6 Method for determining the activity of glucose 6-phosphate dehydrogenase enzyme**

By measuring the rate of change of absorbance due to the reduction of oxidized nicotinamide adenine dinucleotide phosphate (NADP+) in the sample under the influence of glucose-6-phosphate (G-6-P) at a wavelength of 340 nm, and G6PD was used for research (Spinreact, Cromakit, S.L., Granada, Spain) set of reagents was used.

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{G-6-PDH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^{+}$$

Red blood cells are diluted in a 1:6 ratio (R4 Digitonin) biological detergent and stored at a temperature of + 4 °C. It was kept for 15 min, then spun in a centrifuge at 3000 rpm for another 10 min and suspended suspended. To the supernatant extract, 0,5 ml of 0,1 M triethanolamine (TEA) buffer, pH 7.6 (triethanolamine (TEA) buffer), 15 µl of nicotinamide adenine dinucleotide phosphate ($NADP^{+}$) solution in

the oxidized form was added and kept at 37 °C. was incubated for 5 min in a thermostatic bath at room temperature. After incubation, glucose-6-phosphate substrate solution was added and the activity of glucose-6-phosphate dehydrogenase enzyme was determined. The reaction was initiated after the addition of $NADP^+$ reagent. Glucose-6-phosphate is first oxidized to gluconate-6-phosphate and nicotinamide adenine dinucleotide phosphate (NADPH) with the presence of the enzyme glucose-6-phosphate dehydrogenase. The amount of regenerated, i.e. reduced, nicotinamide adenine dinucleotide phosphate (NADPH) corresponds to the amount of glucose -6-phosphate and, accordingly, to the amount of glucose.

The activity of G-6-PDH was calculated by subtracting the activity of PGD from the total activity of the enzyme. Enzyme activity is expressed as μmol/min/g Hb. A unit of enzyme activity is defined as 1 μmol of NADPH formed per minute per gram of hemoglobin.

To calculate G-6-PDH activity as mU/g haemoglobin use the following equation:

$$G-6-PDH = \frac{\text{mU.erthrocytes per mL x 100}}{\text{Hb}\left(\frac{g}{dL}\right)} \quad (5)$$

here, 100 = Factor to convert from mL to dL

Hb (g/dL) = Haemoglobin concentration determined for each specimen

### 2.3.7 Method for determining the concentration of reduced (GSH) and (GSSG) oxidized glutathione

Glutathione disulfide (GSSG) and reduced glutathione (GSH) levels were measured in the erythrocyte fraction by a fluorimetric method using a microplate fluorescence reader (*FLx800; BioTek Instruments Inc., Highland Park, VT, USA)* at wavelengths between 340 and 420 nm [176].

This method is based on the specific reaction of reduced glutathione (GSH) with orthophthalic aldehyde (OPT) at pH 8.0, resulting in a highly fluorescent product activated by emission peaks at 350 nm and 420 nm. The reaction is dependent on the final pH, which should be 8.0. Below pH <8.0 the fluorescence intensity decreases and above pH >8.0 the conversion of GSSG to GSH occurs. That is, 300 μL of erythrocytes were suspended in sodium phosphate buffer (A) (10 mmol/L sodium phosphate, 5 mmol/L EDTA-$Na_2$, pH=6.25) in a 1:1 ratio. Then, 270 μL of sodium phosphate buffer (A) (10 mmol/L sodium phosphate, 5 mmol/L EDTA-$Na_2$, pH 6.25) was added to 30 μL of diluted erythrocytes, 300 μL was deproteinized in ice-cold 10% TCA, and centrifuged at 20,000g for 15 minutes. In addition, a 10:1 standard curve solution of GSH reagent in sodium phosphate buffer (B) (100 mmol/L sodium phosphate, 5 mmol/L EDTA-$Na_2$, pH=8.0) was prepared. To measure glutathione in the supernatant, samples were incubated in sodium phosphate buffer (B) (100 mM sodium phosphate, 5 mM EDTA-$Na_2$, pH 8,0) and OPT (orthophthalaldehyde/ethanol, 1 mg/ml) solution at 25°C for 10 minutes and the fluorescence of the samples was measured. To determine the amount of glutathione, the fluorescence of the samples is compared to the curve of the GSH standard

solutions (0.2 to 3.25 nmol/mL) GSH values are expressed as µmol/g Hb. The reaction depends on the final pH, which should be pH=12.00. Fluorescence intensity below this value is due to the reduction of glutathione (GSH) produced by the enzyme glutathione reductase. To prevent this, glutathione reductase in the model is inhibited by N-ethylmaleimide (NEM). For the measurement of oxidized glutathione (GSSG), 200 µL of the supernatant is preincubated in 80 µL of N-ethylmaleimide solution (NEM 5 mg/mL) for 40 minutes. To prevent oxidation of GSH to GSSG, N-ethylmaleimide was added to the samples, which were then alkalinized with 0.1 N NaOH solution.

The concentrations of reduced and oxidized glutathione were measured using a standard curve, and their ranks were expressed as nmol/mg protein. The GSH: GSSG ratio was determined using the formula (GSH-GSSG)/GSSG). Results were calculated using a four-parameter logistic fit and expressed as µmol/g Hb.

### 2.3.8 Determinating of glutathione peroxidase (GPx) activity in red blood cells

GPx activity in red blood cells is indirectly measured by a coenzymatic reaction. The determination of GPx activity is carried out by a coenzymatic reaction in which glutathione (in its reduced form) and NADPH react with cumene hydroperoxide to form oxidized glutathione and cumene hydroxide (GPx enzyme catalyzes the oxidation of glutathione (GSH) with cumene hydroperoxide) [177-178]. The GPx enzyme catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. Glutathione reductase (GSH) an glutathione (GSSG) oxidized in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) is immediately converted to its reduced form with the oxidation of NADPH to $NADP^+$, and thus the decrease in absorbance at a wavelength of 340 nm was measured using a spectrophotometer.

$$\text{R-OOH} + 2\ \text{GSH} \xrightarrow{\text{GPx}} \text{R-OH} + \text{GSSG}$$

GPx activity in whole blood was measured in units per liter of hemolysate according to the test procedure supplied with the kit. Since the GPx enzyme is inside red blood cells, it is diluted in Hemolysis buffer (Phosphate - EDTA 10 mM, pH=6,25, EDTA-$Na_2$ 1 mM) at a ratio of 1:20, then it is suspended in a centrifuge at 20000 g for 15 minutes. For GPx analysis, a solution containing reduced glutathione, glutathione reductase and NADPH is added to the sample. To evaluate GPx activity in the supernatant, 10 ml of sodium dihydrogen phosphate buffer ( Phosphate Buffer - EDTA, 100 mM, pH=7,5, EDTA-$Na_2$ 1 mM), 1,7 mg of NADPH reaction mixture, solution A not catalyzed reaction and 2,6 mg of $NaN_3$, from 10 ml of sodium dihydrogen phosphate buffer (Phosphate Buffer - EDTA, 100 mM, pH=7,5, EDTA-$Na_2$ 1 mM ), 12,3 mg of glutathione, 1,7 mg of NADPH, 11 µl of glutathione reductase (GRd) reaction mixture B solution was used for catalyzed reaction, 25°C for 5 min was incubated. Enzymatic reaction was started by addition of cumene hydroperoxide substrate and all measurements were measured by a plate spectrophotometer *BioTek Power-Wavex Microplate Scanning* instrument at a

wavelength of 340 nm in 3 minutes to measure how much the optical activity decreased [179]. The rate of decrease in absorbance is directly proportional to the GPx activity in the sample.

### 2.3.9 Determination of glutathione reductase (GRd) activity in red blood cells

The Cayman's glutathione reductase (GRd) enzyme activity assay kit *(703202; Cayman Chemical, Ann Arbor, MI, USA*) measures GR activity by measuring the rate of NADPH oxidation:

$$GSSG + NADPH + H^+ \xrightarrow{GRd} 2GSH + NADP^+$$

NADPH to $NADP^+$ oxidation at 340 nm of absorption with a decrease with goes and to GRd activity in the sample directly proportional.

Glutathione enzyme activity of NADPH oxidation optically due to the regeneration of GSH by density with a decrease was evaluated.

Since the GRd enzyme is inside red blood cells, it was diluted with $H_2O$ in distilled water at a ratio of 1:20, and then suspended in a centrifuge at 10,000 g for 15 minutes. The prepared supernatant was diluted 1:20 in the dilution buffer (*GRd Sample Buffer*). To assess the activity of GRd in aliquots, pour spectrophotometer 96 well plates containing 100 µl of room temperature Potassium-phosphate buffer (Assay Buffer pH 7,5), 20 µl of aliquot, 20 µl of reduced glutathione (GSSG), 50 µl of NADPH, and immediately use the tablet spectrophotometer *BioTek Power-Wavex Microplate*. The optical activity at 340 nm wavelength was measured during 3 minute incubation with a scanning device. Plate samples contained NADPH (0,38 M) and GR (3,5–20 nM) to avoid interference with NADPH oxidase activity. The reaction was initiated after adding the GSSG reagent to the samples on the tablet.

### 2.3.10 Determination of lipid oxidation in plasma

LPO was determined by a commercial colorimetric kit (KB03002, *Bioquochem kit, BQC Redox Technologies, Asturias, Spain*) that estimates both malondialdehyde (MDA) and 4-hydroxyalkenals, the LPO assay kit measures the concentration of malondialdehyde (MDA) and hydroxynonenal (HNE) as an index of lipid peroxidation [180].

This method is based on the reaction of a chromogenic reagent (N-methyl-2-phenylindole) with malondialdehyde (MDA) and hydroxynonenal (HNE) that forms a stable chromophore with a maximum optical density at a wavelength of 586 nm at a temperature of 40°C. The reactions between aldehydes and indoles are shown below:

$$\text{Reagent A} + \text{MDA} \rightarrow \text{C} \ (\lambda\text{máx} = 586 \text{ nm})$$
$$\text{Reagent A} + \text{HNE} \rightarrow \text{C} \ (\lambda\text{máx} = 586 \text{ nm})$$

Spectrophotometric determination of plasma lipid peroxidation (LPO) levels

was performed in a microplate reader. To a 100 µl aliquot of the plasma sample, 325 µl of Reagent A (N-methyl-2-phenylindole) and 75 µl of Reagent B (methanesulfonic acid (MsOH)) were added and incubated in a boiling water bath at 40°C for 40 minutes and then cooled to room temperature. To prepare Reagent A, lipid peroxide solvent (acetonitrile, methanol) was previously added to N-methyl-2-phenylindole. One malondialdehyde (MDA) reacts with 2 molecules of reagent A to form a stable chromophore with an absorption maximum of 586 nm. Metasulfonic acid reagent B (Metianesulphonic acid) was added as an acidic solvent to simultaneously determine two biomarkers (MDA and 4-HNE). Samples were centrifuged at 15,000 g for 10 min to obtain a clear supernatant, using 1,1,3,3-trimethoxypropane as a standard. Plasma MDA concentrations were calculated using a standard curve. Results are given as nmol/ml [181] and calculated the aldehydes values (MDA+HNE) of the samples using the equation obtained from the linear regression of the standard curve replacing the corrected absorbance values for each sample.

$$(\mathrm{MDA} + \ 4\ \mathrm{HNE}) = \mathrm{A586} - b/a * df \qquad (6)$$

Here,

$A_{586}$ = Net absorbance of the sample at a wavelength of 586 nm;

b = intersection coefficient;

a = regression coefficient (slope);

df = үлгінің сұйылту коэфициенті.

The amount of MDA in plasma was calculated using a standard curve. Results are expressed in nmol/mL.

### 2.3.11 Determination of AOPP (Advanced oxidation protein products) in plasma

Spectrophotometric determination of AOPP level in plasma was performed in a microplate reader using the method of *V. Witko-Sarsat et al* [182, 183]. The principle of the analysis is a colorimetric reaction between plasma proteins and chlorinated oxidants (for example, chloramine-T). The method is based on measuring the level of tyrosine, a specific marker of protein oxidation. In the presence of potassium iodide KI (Potassium iodide) and acetic acid (acetic acid), tyrosine forms a component that absorbs light at 340 nm. This absorbance is measured against known concentrations of chloramine-T, which absorbs at 340 nm under the same conditions. All analyzes were performed in triplicate.

An aliquot of the plasma sample was diluted 1:5 in PBS phosphate-buffered saline, pH 7,4. The absorbance at 340 nm was measured using a standard curve that included potassium iodide (0-100 nmol/ml), 20 µl of acetic acid, and a chloramine-T solution. Before determining the calibration curve (12.5, 25, 50, 75, and 100 mmol/L), the original chloramine-T (10 mmol/L) solution was diluted 100 times (100 mmol/L) in phosphate-buffered saline pH 7,4 (PBS phosphate-buffered saline) diluted. Potassium iodide (KI, 1,16 mol/L) was also prepared in phosphate-buffered saline pH 7,4 (PBS phosphate-buffered saline). Acetic acid (96%) was used in its

original form. Using 340 nm as the starting wavelength, the absorbance on a spectrophotometric plate reader is read in order to determine the AOPP concentration through comparison with a chloramine standard curve.

Using phosphate-buffered saline (PBS) diluted 1:5, 200 μL aliquot of 1:5 plasma and chloramine-T standard samples (0–100 μmol/L) were added to a 96-well UV chamber. After adding 10 μL of 1.16 M KI to the standard samples, all samples were treated with 30 μL of acetic acid after two minutes. The samples were then analyzed at 340 nm using a BioTek Power-Wavex Microplate Scanning Plate Spectrophotometer. The unit of measurement for AOPP concentrations is chloramine-T equivalents or nmol/ml.

### 2.3.12 The method of determining the amount of cytokines by immunoenzymatic analysis

To analyze the expression profile of cytokines IL-6, IL-10, IL-18, TNF-α in the plasma fraction (Milliplex) HCYTA-60K-03 Human Cyto Panel A was carried out using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. A mixture of 25 μl diluted plasma aliquots diluted 1:4 in sample-specific diluent and 25 μl reference samples were added to each well. The cell surface was covered with film after 25 μl of beads tagged with particular antibodies against each cytokine were added.

They were incubated at 20–25°C for two hours. Following incubation, the films were taken off the plate, cleaned three times with 200 μl of washing buffer, and filtered until dry. Sample analytes attach themselves to antibodies that have been immobilized on the working electrode's surface. After the analyte in the sample binds to the antibody on the surface of the microspheres, a biotinized detection antibody is introduced into the complex, the plate is again covered with film and placed in a shaker for 1 hour.

Then, to complete the reaction on the surface of each microsphere, the reaction mixture (beads) was incubated with 25 μl streptavidin-phycoerythrin solution (conjugate) for 30 min at room temperature and washed again three times.

The beads were resuspended in 150 μL of Sheath Fluid PLUS or Drive Fluid (find out why) and the concentration of each cytokine was determined using the Luminex® xPONENT® program on a Luminex 200 (Bio-Rad Laboratories, Madrid, Spain). A parallel standard curve was drawn for each cytokine. Cytokine levels are expressed in pg/ml.

### 2.3.13 Isolation of total RNA (including microRNAs)

Total RNA isolation was carried out from 200 μl of blood plasma using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Werfen España, ref. 217204) according to the following protocols: 90 μl of RPL buffer containing guanidine dithiocyanate was added to the plasma, together with RNase and protein complexes and a detergent to facilitate the denaturation necessary for cell lysis in the plasma,vortexed for 5 s and incubated at room temperature for 3 min. After 3 min, 30 μl of RPP buffer was added to the tube to precipitate the proteins. Vortex for 20 seconds and incubate for 3 minutes at room temperature. Proteins were centrifuged at

12000 g for 3 min at 21℃ (room temperature) to precipitate proteins. Above the precipitate, 310 µl of clear, colourless supernatant containing RNA was transferred to a new microcentrifuge tube. The silica gel membrane column was vortexed with 310 µl of isopropanol in a 1:1 ratio to ensure proper binding conditions for all RNA molecules from approximately 18 nucleotides. Buffer solution with ethanol or isopropanol was added to bind the sample. 620 µl of binding solution was transferred to RNeasy UCP MinElute Spin columns, and the spin columns were placed in a centrifuge at 8000 g for 15 seconds. Then, add 700 µl of RWT buffer to an RNeasy UCP MinElute Column tube to wash the supernatant and centrifuged at 8000g for 15 seconds. After 15 seconds, the water underneath was drained. The flow section was removed for washing, and washing buffer was added to the column. The column was centrifuged again to push the wash buffer through the membrane. This removes any impurities remaining on the membrane, leaving only the nucleic acid bound to the silica gel. Then 500 µl of RPE buffer was added and centrifuged at 8000 g for 15 s. Then 500 µl were washed with 80% ethanol and centrifuged at 8000g for 2 minutes. Then 500 µl was washed with 80% ethanol and centrifuged at 8000 g for 2 min. The spin tubes from the centrifuge were placed in a filter clean RNeasy kit tube and centrifuged for 5 minutes with the lid open at maximum speed. To elute total RNA, 20 µl of RNase Free Water was added to the supernatant tube. For elution, the wash buffer is removed and elution buffer (or plain water) is added to the column. The tube was centrifuged again and the elution buffer was passed through the membrane. The elution buffer removes the nucleic acid from the membrane, the nucleic acid was collected at the bottom of the tube and the RNA was stored at -80°C. *Qubit 3.0* ( *Thermo Fisher Scientific* ) to determine the concentration of extracted microRNA and *Nanodrop 2000* (*Thermo Fisher Scientific* ) was used to determine the quality of the assay.

### 2.3.14 cDNA extraction and reverse transcription PCR

Total RNA was extracted using a specific primer for each included miRNA according to the *TaqMan Advanced miRNA Assays* protocol for quantitative analysis after isolation method and quality control.

By adding Poly(A) reaction mixture (3 µl) to isolated mature (full-length) miRNA (2 µl) tablets were incubated in iQ5 thermocycler at 37°C for 45 min, then at 65°C for 10 min, modified by extending the 3' end (tail), and stored at 4°C. The 5' ends were then extended in a thermocycler at 16°C for 60 min with adapter ligation reaction mixture in a final volume of 15 µl.

Reverse transcription of microRNAs to cDNA was performed using the TaqMan miRNA reverse transcription kit (Life Technologies, Thermo Fisher Scientific, Madrid, Spain) and specific TaqMan miRNA assays for miR-21, miR-126 according to the manufacturer's recommendations. Plates were incubated in an iQ5 thermocycler at 42°C for 15 min and finally at 85°C for 5 min, then held at 4°C ( Bio-Rad Laboratories, Inc., Hercules, CA, USA). A cDNA amplification mix consisting of 45 µL miR-Amp Primer Mix and TaqMan miR-Amp Master Mix (Life Technologies, Carlsbad, CA, USA), was then prepared and 5 µL reverse transcription mix was added to give a final volume of 50 µL required for target microRNA

recruitment. That is, the modified miRNAs were reverse transcribed in a final volume of 30 µl, and 5 µl of reverse transcription reaction products were amplified using miR-Amp reaction products to obtain homogeneous cDNA.

The mixture was then incubated in iQ5 thermal cyclers under the following conditions: enzyme activity at 95°C for 5 min, denaturation at 95°C for 3 sec; 30 s annealing at 60 °C, 14 amplification cycles and finally 99.9 °C 10 min quenching. The preamplified products were then diluted 1:10 with RNase-free water and used for real-time reverse transcription polymerase chain reaction (RT- PCR ).

### 2.3.15 Real-time quantitative PCR (RT-qPCR) method to determine the expression levels of microRNAs

Quantitative real-time PCR specific to our study was performed using miRNA TaqMan Advanced Assays (miR-21-5p: assay 477975_mir; miR-126-5p: assay 477888_mir; miR-143-3p: assay) using the Agilent Technologies Stratagene Mx3005P system (Agilent Technologies, Spain). 477912_mir) (Thermo Fisher Scientific) was performed using the TaqMan Fast Advanced Master Mix in a final volume of 20 µl. All reactions of quantitative real-time PCR were repeated in triplicate. Limit cycle values were determined using a set threshold, and values below ≥32 (Ct) in thermocyclers were not included in the analysis. Data were analyzed using SDS 2.3 and RQ Manager 1.2 software packages (Life Technologies), and relative gene expression was calculated using the 2-ΔΔCT (ΔCt-target miR-ΔCt-control gene) method. miR-143 plasma microRNA was used as an internal control, and cel-miR-39-3p was used as an external control. The microRNA expression of each patient was calculated relative to the Ct value of the control group on the plate. Reactions were incubated at 95°C for 10 minutes, followed by 40 cycles of 95°C for 20 seconds and 60°C for 1 minute ( second). The obtained results were processed using Step One Plus 2.2.2 and ExpressionSuite 1.0.3 software. Comparative quantitative analysis of microRNA and mRNA is carried out on the basis of indicators of the Ct cycle by comparative Ct.

### 2.3.16 Statistical analysis

Statistical analysis of the obtained data was carried out in IBM SPSS Statistics for MacOS, version 25.0 (WPSS Ltd., Surrey, UK), graphs were constructed using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as arithmetic mean ± (SEM) standard deviation—study in pictures.

Continuous data were tested for normality using the Shapiro-Wilk test, and Levene´s statistic verified the homogeneity of variance.

For data that followed a normal distribution, homogeneity of variances was confirmed using Levene's test. Differences between continuous variables between groups were calculated using ANOVA analysis of variance for multivariate analysis, followed by Tukey's test post-hoc analysis. Bonferroni for variables with equal variances, and T2 de Tamhane for not equal ones. For non-parametric data, differences between groups were calculated by the Kruskal-Wallis test and the Mann-Whitney U test. After adjusting for age and gender for non-normally distributed variables, Kruskal-Wallis one-factor analysis (ANOVA) of variance was used.

Pearson's analysis was evaluated to determine correlations between quantitative variables. A receiver operating characteristic (ROC) curve was constructed to assess the diagnostic value of circulating microRNAs and other markers. Area under the curve (AUC) and 95% confidence intervals (CI) were calculated to determine specificity and sensitivity.

Binomial logistic regression analysis was used to examine the relationship between circulating plasma microRNAs, levels of oxidative and inflammatory biomarkers, and the presence of diabetes and complications. Results are displayed as odds ratios (ExpB) and 95% confidence intervals (CIs). Differences are considered statistically significant at values $p < 0.05$.

## 3 RESULTS AND DISCUSSION

### 3.1 Comparative analysis of biochemical parameters (HbA1c, glucose, insulin, HOMA-IR index, total cholesterol, triglycerides) in the blood of patients with type 2 diabetes mellitus

In order to meet the objectives, 47 (57,3%) males and 35 (44,7%) females took part in this study, for a total of 82 participants. The subjects were divided into three groups: control (n = 30, 14 women and 16 men), T2DM patients without complications (n = 26, 7 women and 19 men), and T2DM with complications (n = 26, 14 women and 12 men). During medical examination, all patients underwent a standard general clinical examination, including a questionnaire and self-questioning of the subject, physical examination and anthropometric measurements. During the study of anamnesis, attention was paid to information about the duration of the disease, hereditary burden, and the nature of the therapy.

Table 3 - Anthropometric indices in the control group, in patients with type 2 diabetes mellitus (T2DM) without cardiovascular complications and in patients with cardiovascular complications.

| Parameters | CG (n = 30) | T2DM NC (n = 26) | T2DM + C (n = 26) | p-value |
|---|---|---|---|---|
| Age (years) | 49,64 ± 1,78 | 53,54 ± 2,00 | 55,31 ± 1,91 | |
| Gender (women/men) | 14/16 | 7/19 | 14/12 | |
| Weight (kg) | 77,61 ± 3,57 | 87,50 ± 3,77 | 78,12 ± 3,19 | |
| BMI ($kg/m^2$) | 27,90 ± 0,77 | 30,57 ± 1,25 | 29,49 ± 1,28 | |
| Waist circumference (cm) | 94,5 ± 3,03 | 104,96 ± 2,78 | 103,34 ± 2,21 | |
| Systolic pressure (Pa) | 121,44 ± 2,9 | 132,69 ± 3,9 | 139,65 ± 5,1** | [b] p <0,01 |
| Diastolic pressure (Pa) | 78,1 ± 1,4 | 85,3 ± 2,1 | 84,8 ± 2,7 | |
| Years of diabetes | - | 7,85 ± 0,76 | 11,69 ± 1,5 | |

CG: Non. T2DM NC: T2DM without complications. T2DM + C: T2DM with complications. [a]p—T2DM NC vs. CG in the post-hoc analysis. [b]p—T2DM + C vs. CG in the post-hoc analysis. [c]p—T2DM + C vs. T2DM NC in the post-hoc analysis.

Table 3 shows the profiles and anthropometric parameters of the study participants. The average age of the patients was between 50 and 55 years. Smokers were outnumbered by nonsmokers among all participants, but there were no group differences, and no everyday drinkers were seen. 92% of patients in the experimental group have a family history of type 2 diabetes. All participants had a body mass index (BMI) greater than 25, were overweight or obese, and had no statistically significant differences in BMI, gender, or age between the three groups. In the control

group, average body weight was 23.3%, first-degree obesity - 26.7%, second-degree obesity - 10%, first-degree overweight - 23.3%, second-degree overweight - 16.7%. Additionally, normal body weight was 11.5%, class I obesity was 15.4%, class II obesity was 15.4%, class III obesity was 11.5%, class I overweight was 19.2%, and degree II overweight was 27.9% in the T2DM group without complications. Within the diabetic complications group, normal body weight was 19.2%, first-degree obesity was 30.8%, third-degree obesity was 11.5%, first-degree overweight was 23%, and second-degree overweight was 15.4%. This illustrates how obesity and overweight are common issues in the Mexican population. A significant increase in systolic pressure was observed in patients with cardiovascular complications compared to the control group. This is because hypertension (high blood pressure) was recorded in 73% of the group of patients with T2DM with complications (Table 4). The fact that patients in this group received their type 2 diabetes diagnosis eleven years prior was considered.

Table 4 - BMI classification of study participants

| Parameters | CG (n = 30) | T2DM NC (n = 26) | T2DM + C (n = 26) |
|---|---|---|---|
| Normal body weight (18,5<BMI<24,9 kg/m$^2$), n (%) | 7 (23,3) | 3 (11,5) | 5 (19,2) |
| I degree of overweight (25< BMI <29,9 kg/m$^2$), n (%) | 7 (23,3) | 5 (19,2) | 6 (23) |
| II degree of overweight (25< BMI <29,9 kg/m$^2$), n (%) | 5 (16,7) | 7 (27) | - |
| I degree obesity (30< BMI <34,9 kg/m$^2$), n (%) | 8 (26,7) | 4 (15,4) | 8 (30,8) |
| II degree obesity (35< BMI <39,9 kg/m$^2$), n (%) | 3 (10) | 4 (15,4) | 4 (15,4) |
| III degree obesity (BMI ≥40 kg/m$^2$), n (%) | - | 3 (11,5) | 3 (11,5) |

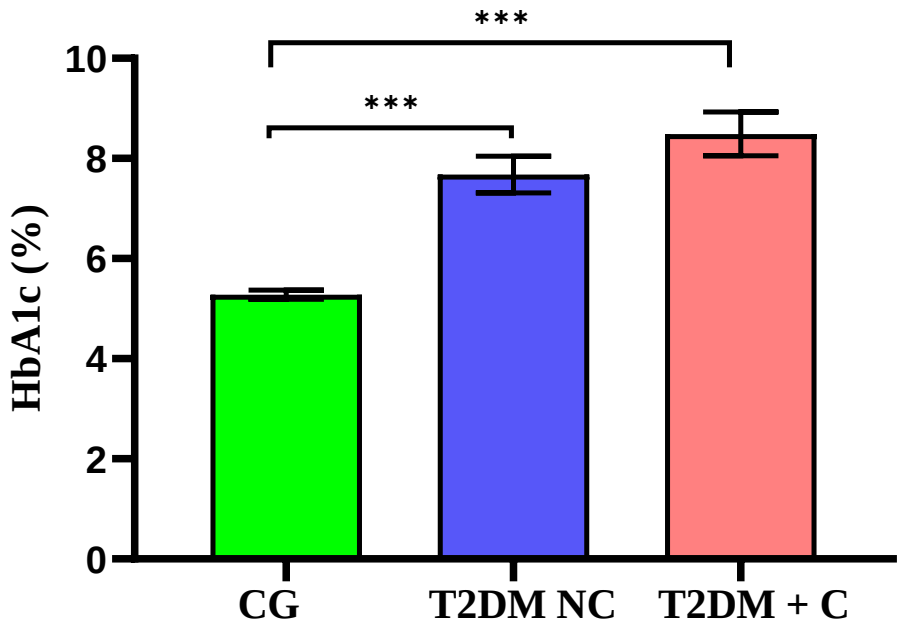
Patients with complications presented with significantly higher systolic pressure, which is to be expected in this type of patient. The disease duration in this group after diagnosis was more than 11 years.

During the work, six indicators were given importance from among the results of biochemical indicators. The first indicator is HbA1c.

Hemoglobin is a complex iron-containing protein in red blood cells and provides oxygen transport from the lungs to the tissues. There are two types of normal hemoglobin: hemoglobin A1 - HbA ($\alpha 2\beta 2$) and hemoglobin A2 - HbA2 ($\alpha 2\delta 2$). In circulating blood, 97% of the heme-containing protein is in the A1 type and only 3% in the A2 type. Glycosylated hemoglobin is formed during the spontaneous binding of hemoglobin A to glucose in the blood.

Glycosylated hemoglobin is an important indicator in the diagnosis of diabetes, which shows the glucose level in the blood over a long period. Unlike sugar levels, glycosylated hemoglobin does not change when taking medications, drinking alcohol, or after exercising, which means the test results remain accurate.

The degree of saturation of HbA with glucose and the rate of their association with each other directly depend on its average content in the bloodstream over the past 120 days (this is the duration of the existence of red blood cells). Finally, several subtypes of glycosylated hemoglobin are formed, the most important of which is HbA1c. This glycohemoglobin variant predominates in venous blood and is related to the degree of hyperglycemia. The formation and disappearance of hemoglobin A1c is a continuous process as new, young red blood cells replace old, dead ones in the bloodstream every day. Based on the results obtained during the determination of glycosylated hemoglobin, it helps to control the development of diabetes better, adjust the dose of hypoglycemic drugs, and prevent the development of serious complications of this disease. Compared with the control group, 5,28 ± 0,09 %, glycated hemoglobin increased to 7,68 ± 0,37 % in the group without complications, and HbA1c was significantly higher in the type 2 diabetes group with complications 8,49 ± 0,44 % ($p < 0,001$) (Figure 6).




Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: HbA1c (%). Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. ***P < 0,001.

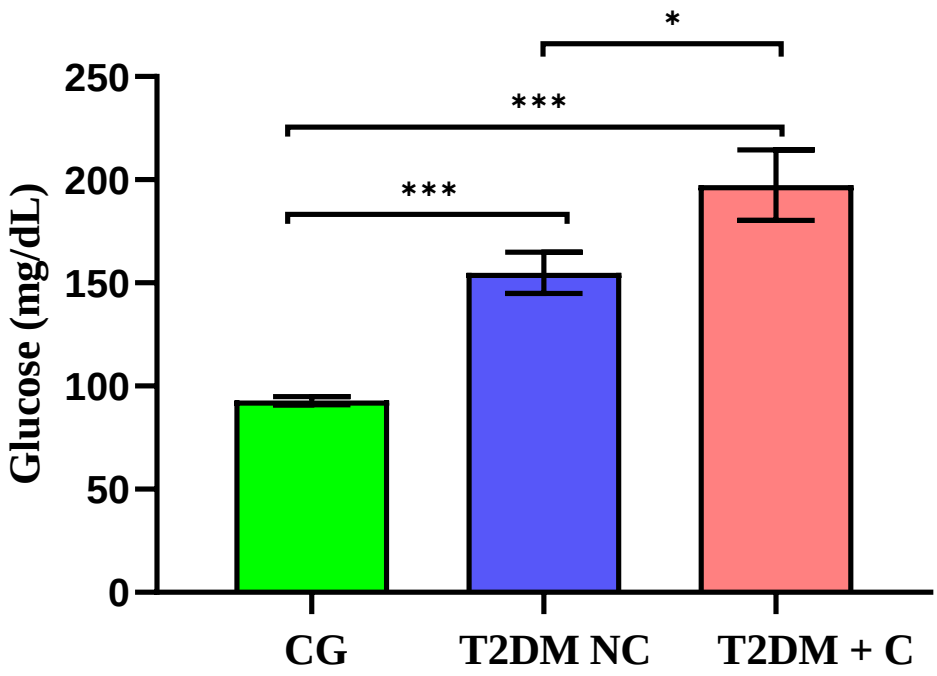
Figure 6 - The comparative level of HbA1c in the blood plasma of patients with type 2 diabetes mellitus.

The HbA1c content of less than 5,7% indicates that there is currently no chance of developing diabetes mellitus. With glycated hemoglobin values in the range of 5.7 to 6,5%, there is no evidence of diabetes; there are manifestations of prediabetes. The

risk of total mortality and mortality from stroke and myocardial infarction increases. The concentration of glycosylated hemoglobin greater than 6,5% indicates the need to exclude or confirm insulin-dependent or insulin-independent diabetes mellitus. Further research methods are needed.

An increase in the level of glycated hemoglobin in the blood always indicates the presence of a long period of hyperglycemia and an increased risk of complications such as retinopathy, nephropathy, polyneuropathy, and micro- and macroangiopathy. A patient suffering from hyperglycemia should strive to achieve a healthy level of glycated hemoglobin in a healthy person – 5,7%. However, this is not always possible. In such cases, therapy aims to reduce the HbA1c concentration to 6.5%. If this goal is achieved, it can be argued that diabetes is well compensated, and the likelihood of the consequences of hyperglycemia in the form of various complications is minimized.

During the study, glucose content in the control group was 92.95 ± 2.02; and in the group without complications it was 154.87 ± 10.01, and in the group with complications it was 197.47 ± 17.10 compared to the group with type 2 diabetes (***p< 0.001) was found to be higher and also showed a significant difference between the two diabetic groups (*p<0.05) (Figure 7).




Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: glucose mg/dL. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. *p<0,05; **p<0,01; ***p<0,001.

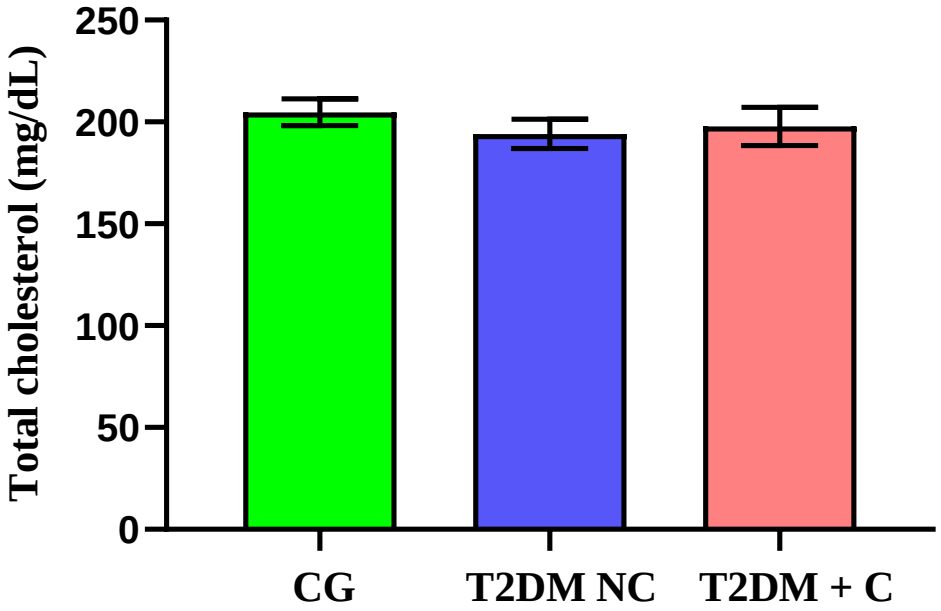
Figure 7 - The comparative level of glucose in the bloodstream of patients with type 2 diabetes mellitus

And measuring the glucose level is not enough to understand how well the

carbohydrate metabolism in the human body is going because the sugar level is constantly changing in healthy people and people with diabetes. Glucose transport to tissues depends on insulin. These tissues are of great importance for the human body, i.e. they are responsible for such important functions as breathing, movement, blood circulation, and the formation of energy reserves from food.

Cholesterol is the main steroid in the human body. It is a vital lipid involved in hormone synthesis and digestive processes and is part of cell membranes. It is produced mainly in the liver and is important for the normal functioning of the body. A disorder in the metabolism of cholesterol (high cholesterol) plays a leading role in the pathogenesis of cardiovascular diseases, contributes to the development of gallstones (cholelithiasis) and increases the risk of pancreatitis.

Total cholesterol was 204.75 ± 6.55 in the control group, 194.12 ± 7.24 in the type 2 diabetes group without complications, and 197.83 ± 9.30 in the type 2 diabetes group with complications, showing no significant change (Figure. 8).



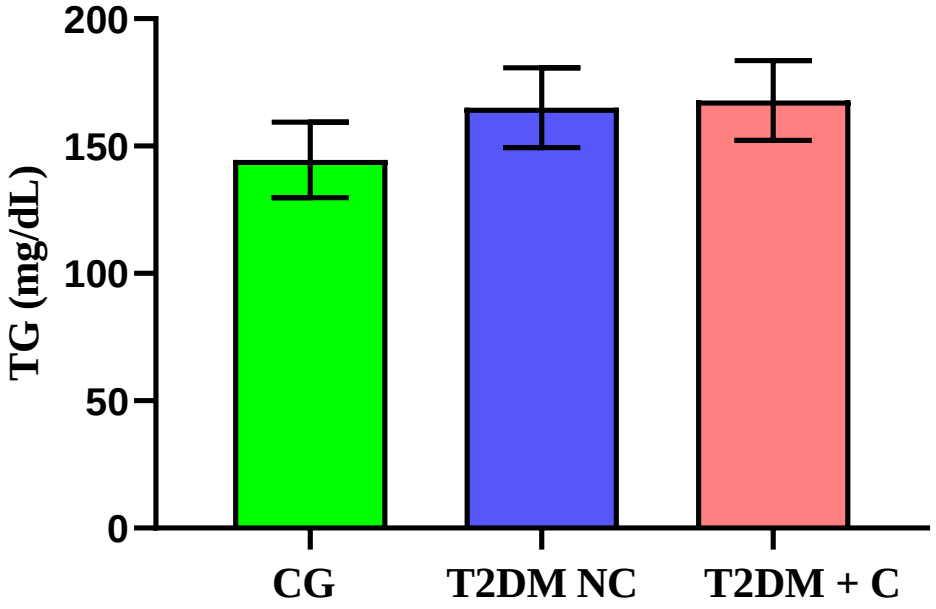

Abscissa axis: CG- controls group, T2DM NC - experimental group of patients type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complication. Ordinate axis: total cholesterol; mg/dL. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs.

Figure 8 - Comparative level of total cholesterol in the blood serum of patients with type 2 diabetes mellitus and normal conditions

This is because the people in the selected groups did not deviate from normal due to a history of cholesterol-lowering biologically active substances.

As for triglyceride, it was 144.52±14.83 in the control group 165.08±15.62 in the type 2 diabetes group without complications; in the group of type 2 diabetes with complications, it showed a value of 167.88±15.7, although it did not show a significant difference compared to the control group but showed a value close to the high value (Figure 9). High triglyceride levels are associated with an increased risk of

heart disease.

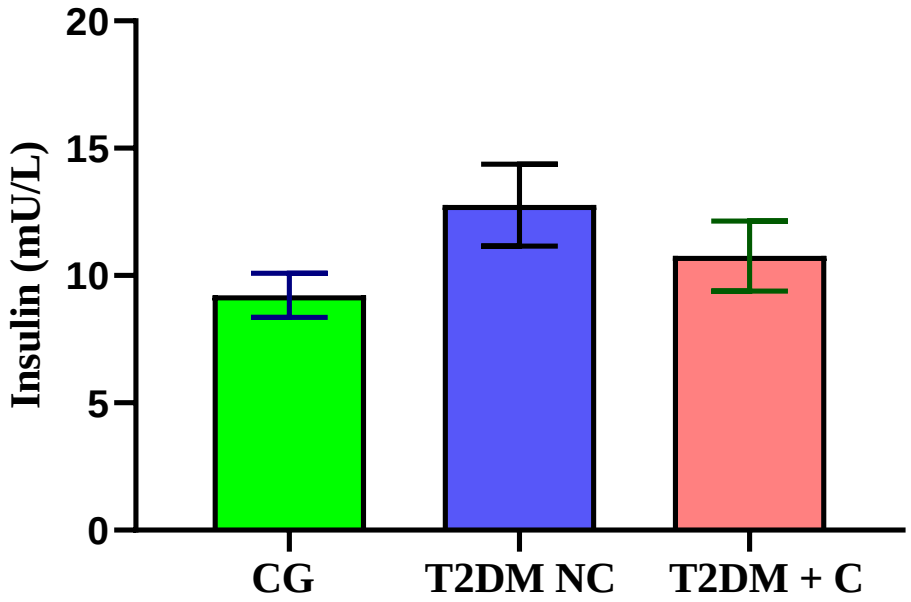



Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: triglyceride mg/dL. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs.

Figure 9 - Comparative measurements of triglycerides in the blood serum between individuals in good health and those with type 2 diabetes

One of the indicators that changes in the blood indicators of diabetes patients is the amount of insulin. Insulin is involved in almost all metabolic processes, but its main function is to affect carbohydrate metabolism. The effect of hormones on these substances is often associated with an increase in excess glucose delivery through cell membranes. As a result, with the help of insulin receptors, an intracellular mechanism is activated, which directly affects the increase of glucose in cells. The mechanism of action of insulin is based on the regulation of the number of membrane proteins that deliver these substances.

It was observed that the insulin level did not change from normal in all groups in the biochemical index. In control group 9,22±0,87 mU/L, in type 2 diabetes group without complications 12,77±1,61 mU/L, in type 2 diabetes group with complications 10,77±1,37 mU/L showed same values (Figure 10). This is because people in the treated groups did not deviate from normal due to a history of daily evening use of antihyperglycemic drugs that lower blood sugar levels.

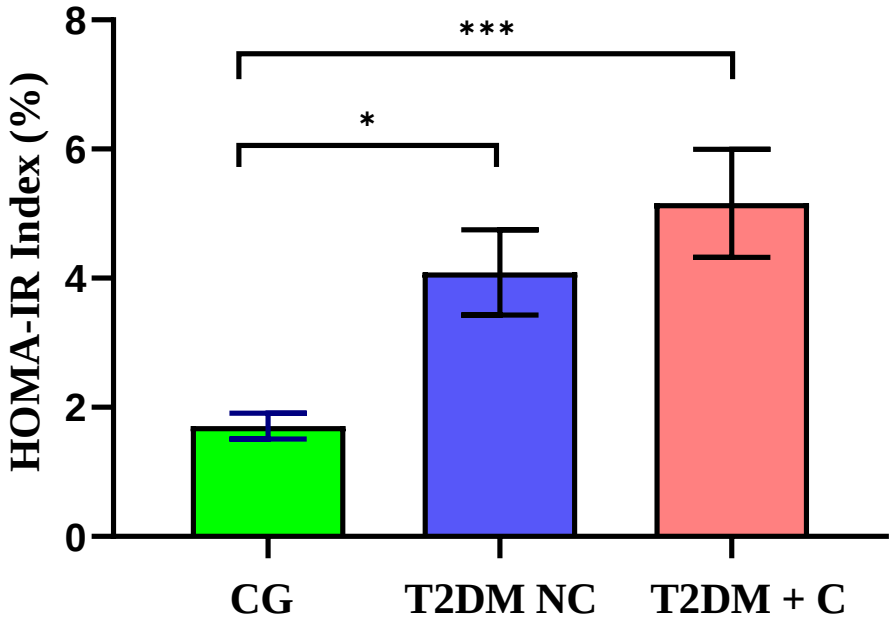



Abscissa axis: CG—controls group, T2DM NC—experimental group of patients with type 2 diabetes without complications, T2DM+C—experimental group of patients with type 2 diabetes with complications. Ordinate axis: Insulin (mU/L). Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs.

Figure 10 - The comparative level of insulin in the blood plasma of patients with type 2 diabetes mellitus.

In type 2 diabetes, the body cannot produce enough insulin. Insulin is a hormone secreted by the pancreas that controls the amount of glucose in the blood. Damage to the pancreas cells leads to a decrease in insulin production, so the cells do not respond to this hormone. This leads to a rapid rise in blood glucose levels. Too little insulin means the body can't absorb glucose from food. When this happens, blood glucose levels rise, and over time, these elevated levels can damage blood vessels and reduce the flow of oxygen- and nutrient-rich blood to the body's organs and nerves. People with type 2 diabetes whose bodies do not respond well to insulin or are insulin resistant may need treatment to help their bodies process glucose better. This helps prevent long-term complications. Adequate insulin sensitivity and glycemic control are important to reduce the risk of diabetes complications.

Significant changes in blood insulin resistance can be observed when evaluating the homeostatic model for insulin resistance. Compared to the control group 1.71±0.20%, without complications 12.77 ±1.61% (*$p < 0.05$) and type 2 diabetes with complications 10.77 ± 1.37% (***$p<0.001$) HOMA-IR index was found to have changed (Figure 11).




Abscissa axis: CG- controls group, T2DM NC - experimental group of patients type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complication. Ordinate axis: HOMA-IR индексі (%). Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. *p<0,05; ***p < 0,001.

Figure 11 - HOMA-IR index in normal conditions and patients with type 2 diabetes

Type 2 diabetes is caused by insulin resistance. The body's tissues (fat, muscle, liver) contain insulin receptors. Insulin acts on these receptors. After the receptors interact with insulin, glucose penetration into the tissues increases dramatically. In the pathology of insulin receptors, their interaction with insulin is disturbed, and tissue resistance to insulin develops. In this case, it is called relative insulin deficiency because insulin secretion is not reduced. In most cases, the function of insulin receptors is observed in obesity. On the other hand, overeating leads to an increase in blood glucose levels. Glucose cannot enter cells because tissues are not sensitive to insulin. It requires a large amount of insulin, so the pancreas begins to produce an excess of insulin, as a result of which β-cells hypertrophy and diabetes develop. The risk of inheritance of type 2 diabetes is 40%. Sometimes type 2 diabetes occurs in teenagers and young adults, and it is 50-80% hereditary. Glycated hemoglobin and HOMA-IR indicators are commonly used to evaluate long-term blood glucose concentration and insulin resistance and predict microvascular and macrovascular complications of T2DM.

Table 5 summarizes the distribution of micro- (retinopathy, nephropathy, and neuropathy) and macro- (stroke, heart failure, and peripheral arterial disease) vascular complications in the T2DM+C group. Different applied therapies are also indicated.

Table 5 – Types of VCs and medications in the T2DM+C group.

| Drug Therapy | Retinopathy (n = 4) | Nephropathy (n = 3) | Neuropathy (n = 14) | CVD (n = 26) |
|---|---|---|---|---|
| | 15,4% | 11,5% | 53,8% | 100% |
| Glucose-lowering medication: inhibitors, GLP-1 receptor | 100,0% | 100,0% | 92,8% | 60,0% |
| Insulin therapy | 50,0% | 0,0% | 57,1% | 80,0% |
| Antihypertensive drugs | 50,0% | 66,6% | 50,0% | 40,0% |
| Cholesterol-lowering therapy | 25,0% | 0,0% | 21,4% | 0,0% |
| Anticonvulsants | 0,0% | 0,0% | 28,6% | 20,0% |

*Antihyperglycemic agents include metformin and second-line glucose-lowering medication (DPP-4 inhibitors, SGLT2 inhibitors, GLP-1 receptor agonists, insulin secretagogues, and thiazolidinediones). Some people need insulin therapy with other antihyperglycemic medications. Painful diabetic neuropathy was treated with anticonvulsants. CVD: Cardiovascular disease. DPP-4: dipeptidyl peptidase-4. SGLT2: sodium-glucose cotransporter 2. GLP-1: glucagon-like peptide type 1.

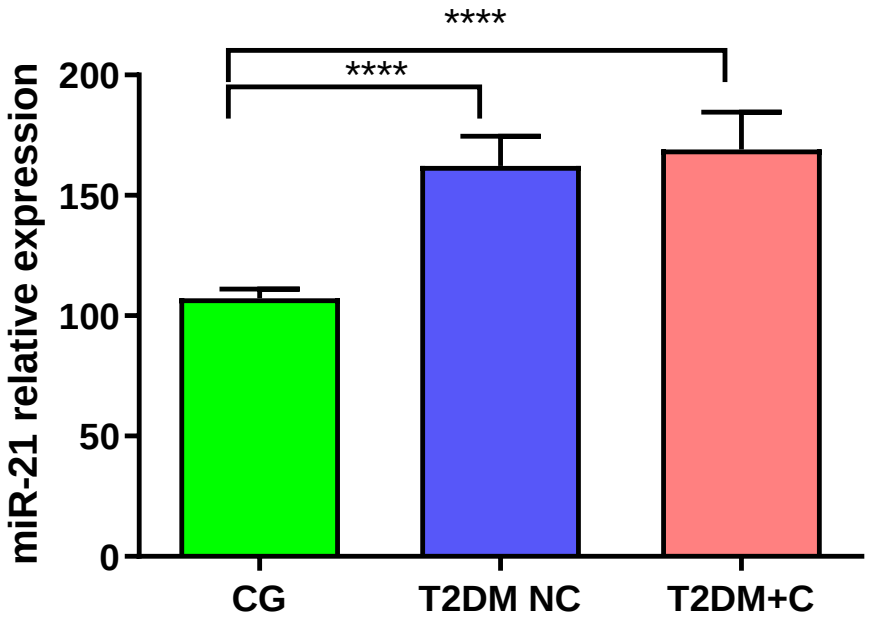
Of the microvascular complications in the group of patients with diabetes mellitus with complications: 15,4% of retinopathy, 11,5% of nephropathy, 53,8% of neuropathy, and 19,2% of macrovascular complications were complications of cardiovascular diseases. During the survey, 100,0% of all diseases caused by complications of retinopathy and nephropathy caused by complications of type 2 diabetes used drugs that reduce the amount of glucose in the body, and only 92,8% of diseases caused by complications of neuropathy, and only 60,0% of diseases caused by cardiovascular complications used only drugs that reduce the amount of glucose in the body. The reason is that they do not determine the amount of glucose in their daily blood. Among these diseases, only those with nephropathy did not use insulin therapy; the remaining 50,0% to 80,0% used it because of insulin resistance and high levels of insulin in the blood. Cholesterol-lowering treatment was used by 25,0% of patients with retinopathy, while only 21,4% of patients with neuropathy used it. And nephropathy and cardiovascular diseases were not used at all. It can be said that this is because they did not pay attention to the treatment provided by the doctor. Anticonvulsants were used only by 28,6% of patients with neuropathy and 20,0% of patients with cardiovascular diseases, indicating that seizures were more common in these diseases. It was found that antihypertensive drugs were used by 40,0% and 66,6% of all complicated diseases; that is, all diseases showed high blood pressure.

In conclusion, according to the results obtained, there are changes in blood indicators of glycosylated hemoglobin, glucose, and homeostatic models of insulin resistance in patients with type 2 diabetes (in groups with and without complications).

**3.2 To identify the relative expression levels of hsa-miR-21-5p and hsa-miR-126-5p in the blood plasma of patients with type 2 diabetes mellitus**

In further studies, two microRNAs (hsa-miR-21-5p, hsa-miR-126-5p) were selected to analyze the expression of circulating microRNAs in the blood plasma of a group of patients with and without complications of type 2 diabetes mellitus. According to the results of other scientific studies, two microRNAs (hsa-miR-21-5p, hsa-miR-126-5p), selected by panel analysis, bioinformatic data (targetscan, mirbase, miRanda) are the targets of these microRNAs as participating genes in the inflammatory process [104].

qRT-PCR results showed that the level of circulating expression of hsa-miR-21-5p in blood plasma was 103,95± 2,93 in the control group compared with 161,25±11,90 in patients with type 2 diabetes mellitus, but without complications. In the group with type 2 diabetes and symptoms of complications, the range of hsa-miR-21-5p expression levels in the blood plasma was 169,06±15,50, that is, compared with the control group with type 2 diabetes without complications and with complications, it significantly increased by group ($p<0,0001$) (Figure 12). These deviations correspond to the deviations in the blood biochemical parameters of glucose and glyceryl hemoglobin.



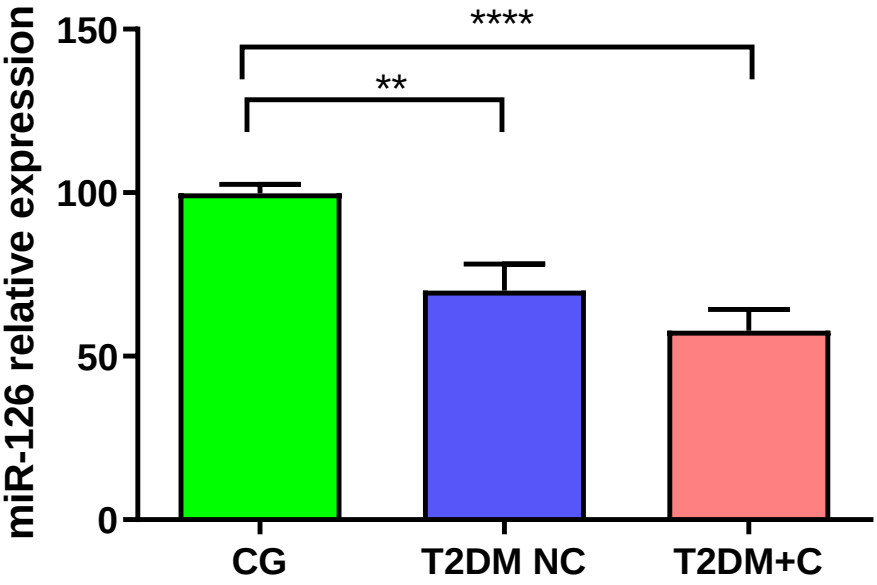

Abscissa axis: CG- controls group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+ C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: hsa-miR-21-5p expression. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. and ****$p < 0,0001$.

Figure 12 – Relative expression level of circulating hsa-miR-21-5p in plasma

In contrast, hsa-miR-126-5p expression in blood plasma of patients with type 2 diabetes without complications compared with control group values, which equal to 101.91 ± 3.26, was decreased to 70.11 ± 8.02 ($p<0.01$).

In addition, the expression level of hsa-miR-126-5p in the blood plasma of patients with complications decreased by 57.84±6.52 ($p <0.0001$) (Figure. 13).

Many studies have shown that specific small molecules of single-stranded RNA are associated with several diseases, such as diabetes and CVD. Recent studies have identified the role of several diabetes-related microRNAs in regulating important metabolic pathways in type 2 diabetes. The discovery that high glucose exposure lowers circulation levels of hsa-miR-126-5p is in line with findings from other investigations [184].

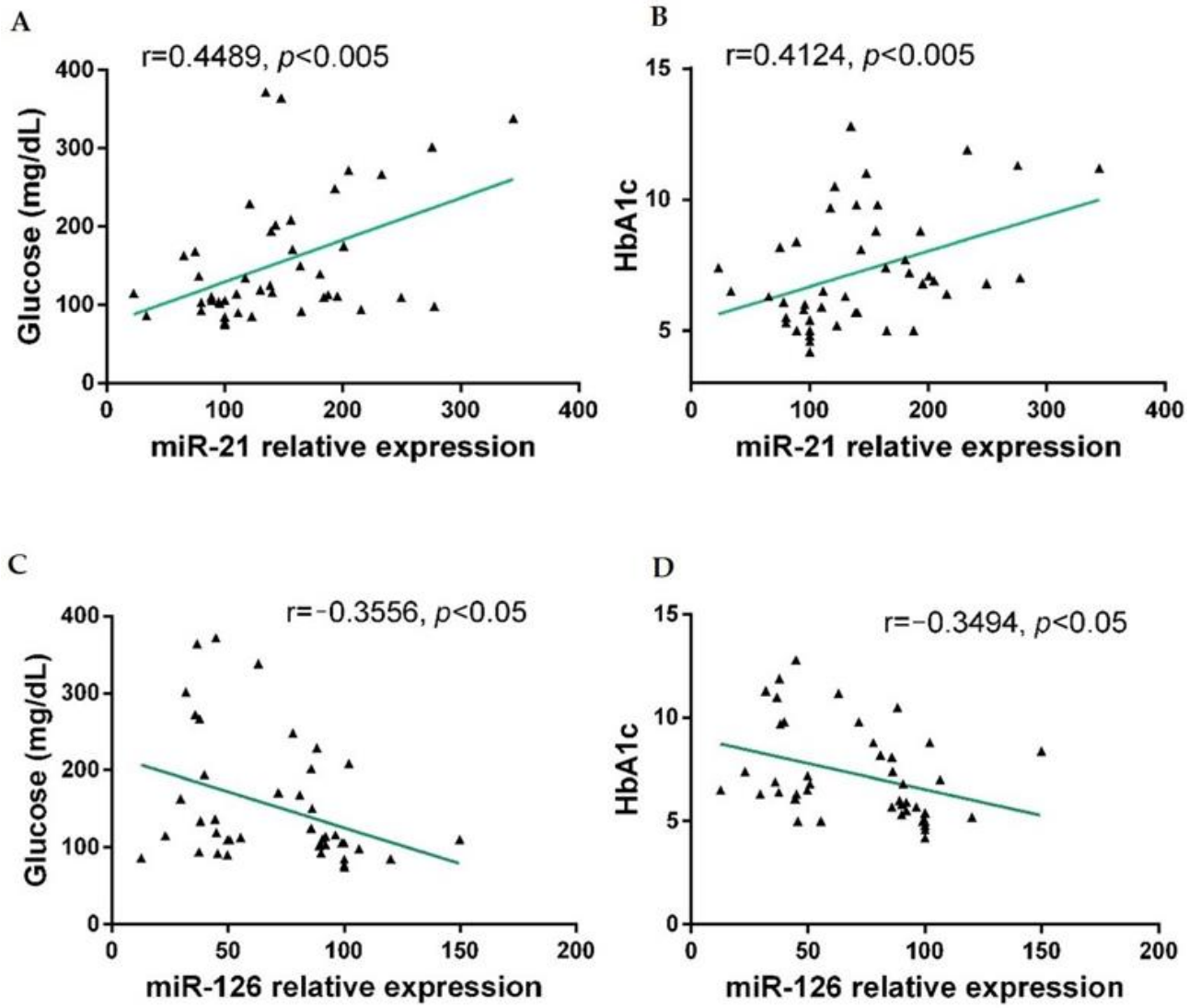



Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - an experimental group of patients with type 2 diabetes with complications. Ordinate axis: hsa-miR-126-5p expression. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. **p <0,01, and ****p < 00001.

Figure 13 – Relative expression level of circulating hsa-miR-126-5p in plasma

In conclusion, the amount of two microRNAs (hsa-miR-21 5p, hsa-miR-126-5p) obtained for the analysis of the expression of circulating microRNAs in the blood plasma of a group of patients with complications of type 2 diabetes mellitus and without complications is presented, which showed a significant difference in a comparative analysis.

In the study, the correlations of relative circulating hsa-miR-21-5p and hsa-miR-126-5p with biochemical parameters in patients with type 2 diabetes were calculated using Pearson correlation coefficient (r) analysis.




Abscissa axis: hsa-miR-21-5p relative expression, hsa-miR-126-5p relative expression. Ordinate axis: HbAc1 – glycosylated hemoglobin %, glucose (mg/dL). Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. **p < 0.01, and ****p < 0.0001. Pearson's correlation coefficient was calculated by the analysis (r). Correlations between relative miR-21 expression and glucose levels (A), miR-21 and HbA1c (B), relative miR-126 expression and glucose (C), and miR-126 and HbA1c (D) were significant.

Figure 14 – Correlations of relative circulating miR-21 and miR-126 with biochemical parameters

The Pearson correlation analysis revealed poor positive correlations between miR-21 expression and glucose (r=0,4489; p<0,005), as well as between miR-21 and HbA1c (r=0,4124; p<0,005) levels (Figure 14 A, B). In contrast, between miR-126 and glucose levels (r=-0,3556; p<0,05), as well as between miR-126 and HbA1c (r=-0,3494; p<0,05), were poor negatively correlated (p < 0.05), (Figure 14 C, D).

Suppose the correlation between two variables is weak, but the p-value (level of significance) is significant. In that case, this means that there is statistical evidence of a relationship between the variables, even if the relationship is weak in practice.

To summarise, it was found that the correlation between hsa-miR-21-5P expression and glucose levels, HbA1c, showed a positive correlation between hsa-miR-126-5P expression and glucose levels, HbA1c gave a negative result. This means that a change in the first value leads to a change in the second value.

### 3.3 To assess the redox status by analyzing markers of oxidative damage in the blood of patients and indicators of the endogenous antioxidant protection system

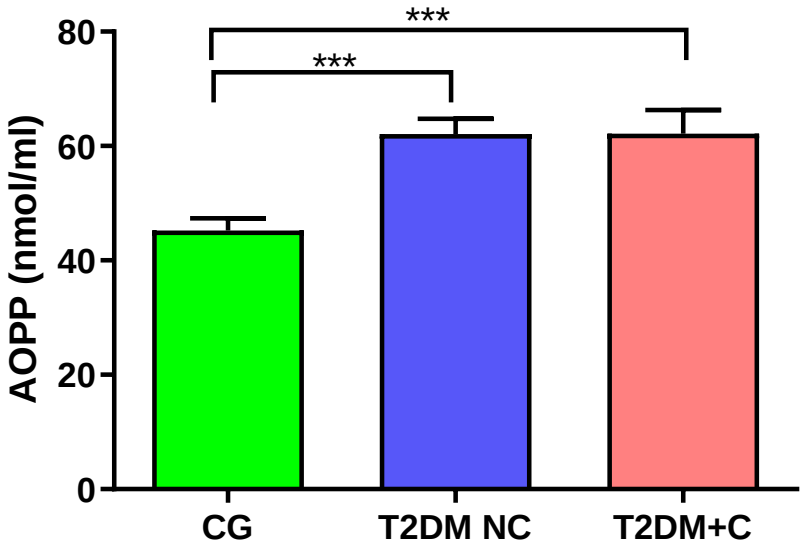
The advanced oxidation protein products (AOPP) assay is a widely published method to determine plasma oxidative stress in plasma based on a spectrophotometric assay in a 96-well microplate format. AOPP has been analyzed in numerous diseases and is widely regarded as an easily measurable marker of oxidative stress [185].

Oxidative stress is a well-established feature of diabetes mellitus and is believed to play an important role in the development of diabetes-related complications [186]. Some studies have found that AOPPs are moderately elevated in adult patients with Type 1 diabetes and more markedly elevated in those with Type 2 diabetes [187, 188], although others have suggested that AOPPs are only significantly increased in Type 2 diabetes [189]. They have been reported to be elevated in Type 2 diabetes [190], and the concentration correlated with insulin resistance [191] or the presence and/or severity of diabetic complications [192], such as retinopathy [193] or nephropathy [194].

Oxidation-reduction reactions are the basic biochemical reactions of the body that maintain its metabolic homeostasis under physiological conditions, change under pathological conditions, and begin to cause damage at the submolecular, molecular, organ, and organism levels. In this regard, the characteristics of the extracellular oxidation state were determined by analyzing the levels of LAT and AOPP in blood plasma. AOPP is formed as a result of carbonylation, a type of oxidation consisting of the processes of glycation and glycoxidation of proteins under the influence of active carbonyl compounds (aldehydes and ketones). AOPP affects the activity of NO, and the level of oxidative modification of proteins is recognized as one of the first signs of damage in various tissues.

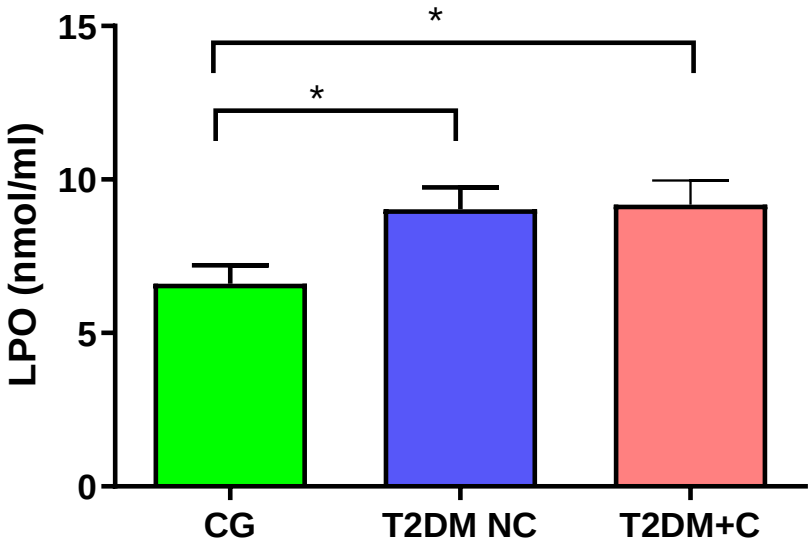
It was observed that the levels of advanced oxidation protein products (AOPP) reached a higher level in the group with type 2 diabetes with 67,62 ± 5,5 and without complications 62,58 ± 2,88 ($p < 0,001$) than in the control group 47,19 ± 2,37 (Figure 15).

Similar to this result, levels of biomarkers of extracellular LAT level were increased in type 2 diabetics without complications 9,03±3,49 and in the group with complications 9,1862±3,98 compared to the control group 6,60 ± 2,44 ($p< 0.05$), (Figure 16).




Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: AOPP-advanced oxidation protein products; nmol/ml. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. ***p<0,001.

Figure 15 - shows the concentration of advanced oxidation protein products in the plasma of patients with type 2 diabetes mellitus and those in normal conditions.



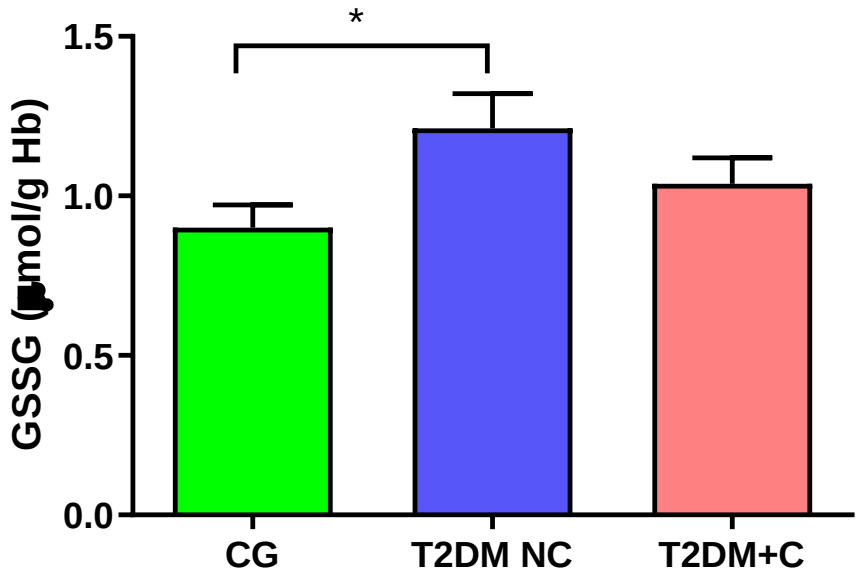

Abscissa axis: CG- controls group, T2DM NC - experimental group of patients type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complication. Ordinate axis: LPO-plasma lipid peroxidation, nmol/ml. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. *p < 0,05.

Figure 16 – LPO levels in plasma of patients with type 2 diabetes mellitus and normal subjects

The study of intracellular oxidative status included measuring antioxidant enzyme activity (CAT, SOD, and G6PD) and glutathione cycle components (GSSG, GSH, GRd, and GPx) in erythrocytes.

Glutathione exists in the reduced (GSH) and oxidized (GSSG) states. Reduced glutathione (gamma-glutamylcysteinylglycine or GSH) is a naturally occurring tripeptide whose reductive and nucleophilic properties play a central role in metabolic pathways as well as in the antioxidant systems of most aerobic cells. In the reduced state, the thiol group of cysteine can donate a reducing equivalent (H++e-) to other unstable molecules such as reactive oxygen species, acting as a natural antioxidant. Glutathione reduces any cysteine disulfide bonds formed within cytoplasmic proteins by acting as an electron donor. Upon electron donation, glutathione becomes a highly reactive molecule and rapidly forms glutathione disulfide (GSSG). This reaction is possible because of the relatively high glutathione concentration in the cells (up to 5 mm in the liver). GSH can be regenerated from GSSG by the enzyme glutathione reductase. In healthy cells and tissues, more than 90% of total glutathione is in the reduced form (GSH), and less than 10% is in the disulfide form (GSSG).

Regarding glutathione cycle components, GSSG levels were significantly higher in the T2DM NC group (1,21±0,67) compared with healthy controls (0,84±0,36 ) ($p < 0.05$) (Figure 17) and There was no difference between the group T2DM NC and the T2DM+C group.



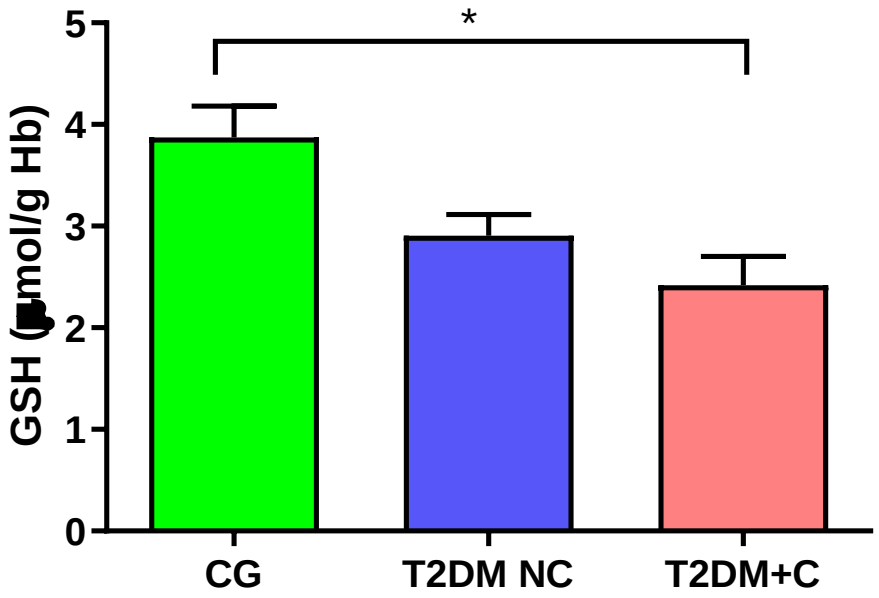

Abscissa axis: CG- controls group, T2DM NC - experimental group of patients type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complication. Ordinate axis: GSSG - oxidized glutathione, µmol/g Hb. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. *p < 0,05.

Figure 17 – Concentration of intracellular oxidized glutathione in the blood of patients with type 2 diabetes mellitus and normal subjects

An increased ratio of GSSG to GSH is considered indicative of oxidative stress. Among other functions of glutathione, GSH plays a critical role as a coenzyme in various enzymes, including glutathione peroxidase, glutathione S-transferase, and thiol transferase. GSH also plays an important role in drug metabolism, detoxifying many xenobiotics and carcinogens, both organic and inorganic.

GSH levels were significantly lower in the T2DM+C group (2,41±0,28) compared with healthy controls (3,87±0,31)), although the GSH level decreased in the group with T2DM+C (2,9±0,20), it did not show a significant difference compared to the control group ($p < 0.05$) (Figure 18).

A decrease in the level of regenerated, i.e., restored, GSH in erythrocyte cells may be associated with a compensatory mechanism of action of antioxidants to combat oxidative stress (initial adaptive reflex reactions aimed at eliminating or weakening functional changes in the body under the influence of inadequate environmental factors).



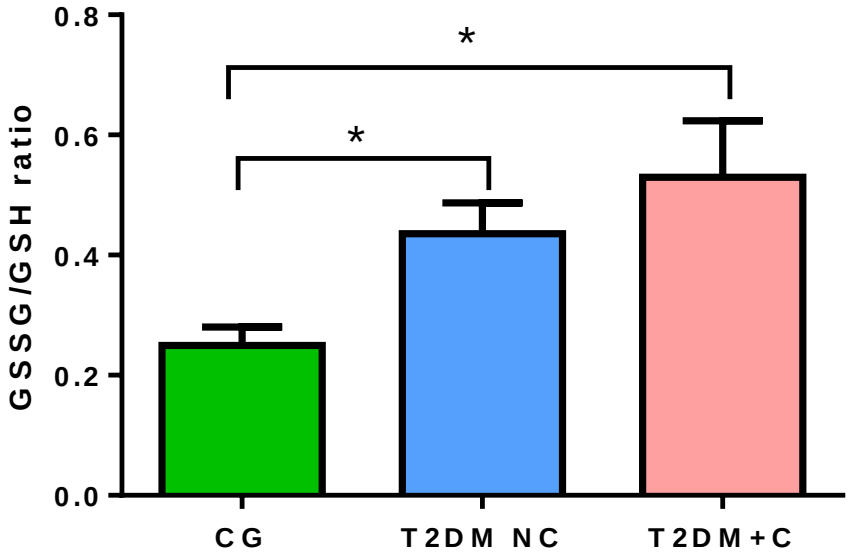

Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: GSH- reduced glutathione, µmol/g Hb. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. *$p < 0,05$.

Figure 18 – Concentration of intracellular reduced glutathione in the blood of patients with type 2 diabetes mellitus and normal subjects

GSH is a multifunctional molecule. Its main role is the antioxidant protection of cells. Besides participating as a cofactor of glutathione peroxidase, GSH itself can nonenzymatically protect cells from free radicals by being a free radical trap due to the presence of a thiol group. The most important marker of antioxidant defenses is the value of the GSH/GSSG ratio (normally 100:1). Its decrease impairs signal

transduction into the cell, gene expression, cell proliferation and differentiation, metabolism, and cell viability [195].

In addition, GSSG/GSH compared with healthy normal participants 0,25 ± 0,14, two experimental T2DM groups without complications 0,44 ± 0,23, and T2DM patients with complications 0,53 ± 0,46 groups, we found that the ratio increased $p<0.05$; (Figure 19).



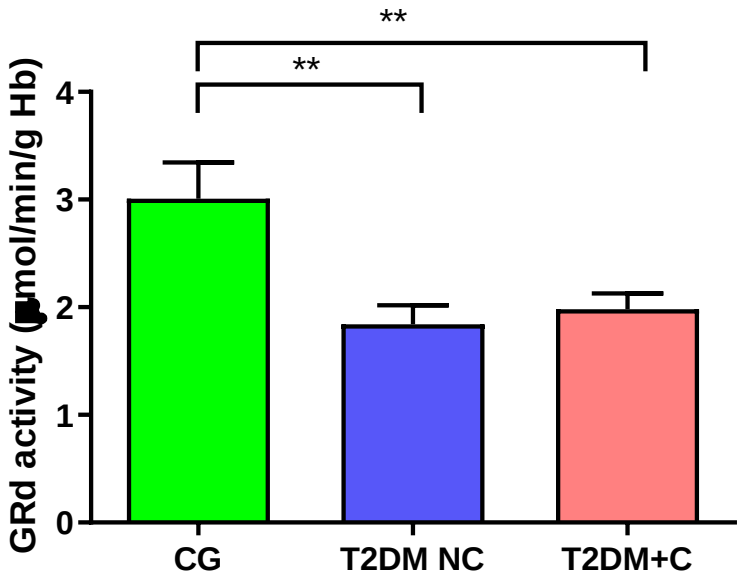

Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: GSSG/GSH ratio. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs. *p < 0,05.

Figure 19 – The ratio of reduced GSH to oxidized GSH (GSSG) in the blood of patients with type 2 diabetes mellitus and normal subjects

According to the study of the Italian scientist Lucia La Sala, this ratio (GSSG/GSH) was significantly increased in diabetic patients compared to the control group. This indicates that the impaired redox state was already present and detected before the onset of clinical manifestations of diabetes. This result was also found in the group of patients with diabetes in this study.

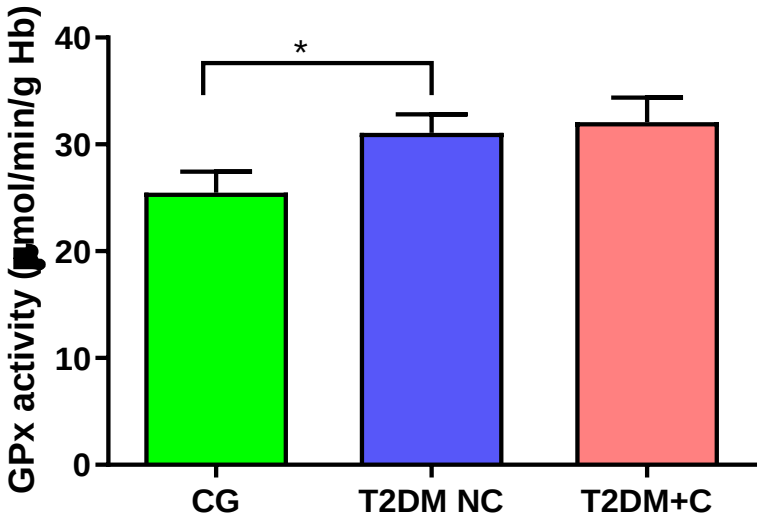
Glutathione reductase is a flavoprotein that catalyzes the NADPH-dependent oxidized glutathione (GSSG) reduction to GSH. This enzyme is essential for the GSH redox cycle, which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is necessary for protection against oxidative stress.

GR for glutathione cycle enzymes was significantly lower in both groups of patients from 3,01±1,30 compared to the control group, i.e., 1,84 ±0,78 with T2T without complications and 1,98±0, 67 with 2TDM with complications, GPx activity did not change (Figure. 20, 21).




Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: GRd - glutathione reductase, µmol/min/g Hb. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs.; **p < 0,01.

Figure 20 – The level of activity of the antioxidant enzyme GRd in the blood of patients with type 2 diabetes mellitus and normal subjects

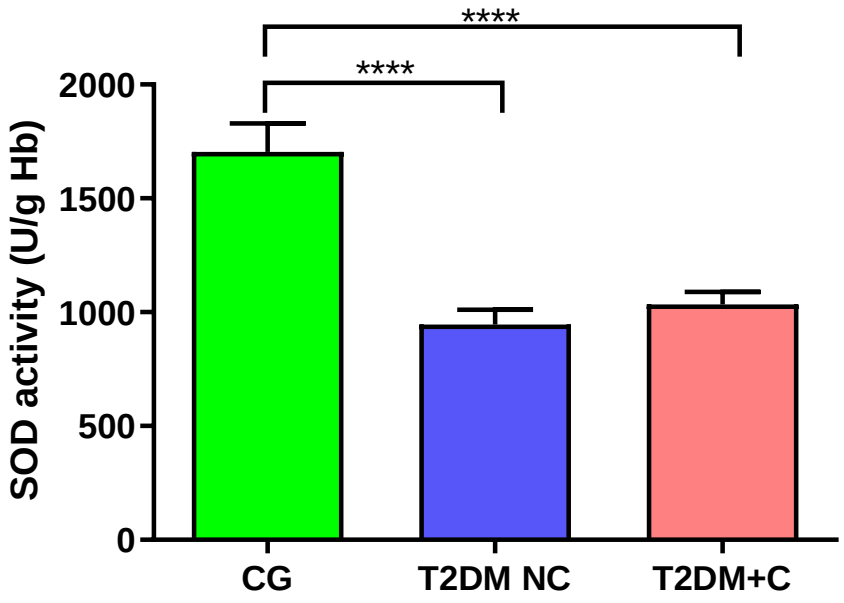



Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: GPx - glutathione peroxidase activity, µmol/min/g Hb. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs *p < 0,05.

Figure 21 – The level of activity of the antioxidant enzyme GPx in the blood of patients with type 2 diabetes mellitus and normal subjects

Superoxide dismutase enhances the dismutation reactions of superoxide radicals generated during biological oxidation. The enzyme ensures the conversion of a superoxide anion radical into a less active oxidizer - hydrogen peroxide, which is inactivated by catalase to two water molecules and an oxygen molecule. With different pathological conditions and in their different periods, both an increase and a decrease in the activity of SOD is possible. Cu-Zn-SOD is mainly found in the cytosol of erythrocytes, the cytoplasm and nucleus of nerve cells, and the intermembrane space of mitochondria [196].

When analyzing the state of antioxidant defense of patients with type 2 diabetes, antioxidant SOD enzymes detected in erythrocyte fractions were significantly reduced in type 2 DM patients with complications (1034,22±54,37) compared to the control group (1034,22±54,37). SOD activity was significant even in patients without complications (945.00 ± 65.88) ($p<0.001$) (Figure 22).



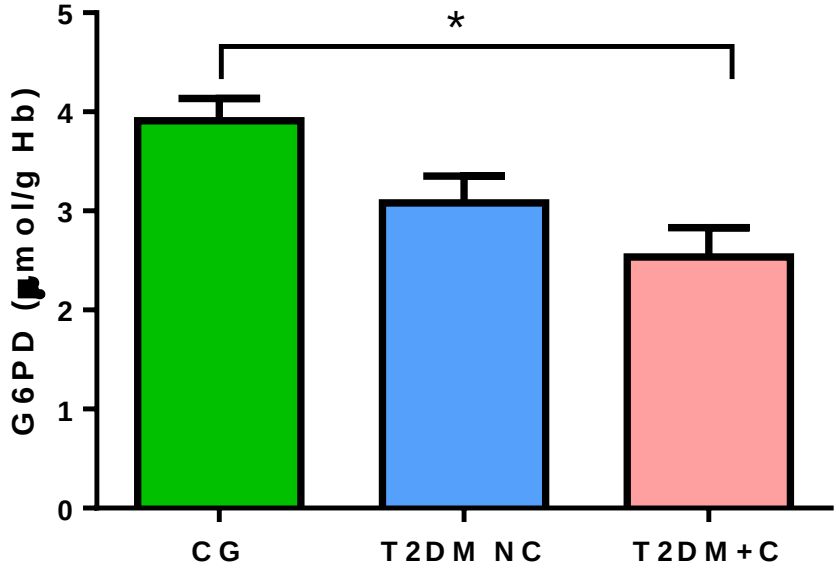

Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: SOD - superoxide dismutase, U/g Hb. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs ****$p < 0,0001$.

Figure 22 - The level of activity of the antioxidant enzyme SOD in the blood of patients with type 2 diabetes mellitus and normal subjects

Glucose-6-phosphate dehydrogenase (G6PD) is a protein molecule that is an important enzyme in glucose metabolism. Manifestations of G6PD deficiency include a wide range of clinical symptoms, the severity of which is often related to the degree of enzyme deficiency. The vast majority of people with G6PD deficiency have no symptoms until the conditions that lead to the onset of the disease occur in the human body.

G6PD showed lower enzymatic activity 2,53±0,3 (p<0,05), in the T2DM group with complications compared to the control group 3,91±0,2 and in the T2DM group without complications 3,08±0,3 compared to the control group there was no significant difference (Figure 23).

Patients with diabetes who experience hyperglycemia experience decreased intracellular NADPH levels and elevated oxidative stress as a result of the suppression of dehydrogenase (G6PD) activity. According to several scientific investigations, the plasma and renal cortex of experimentally diabetic mice showed reduced G6PD activity.



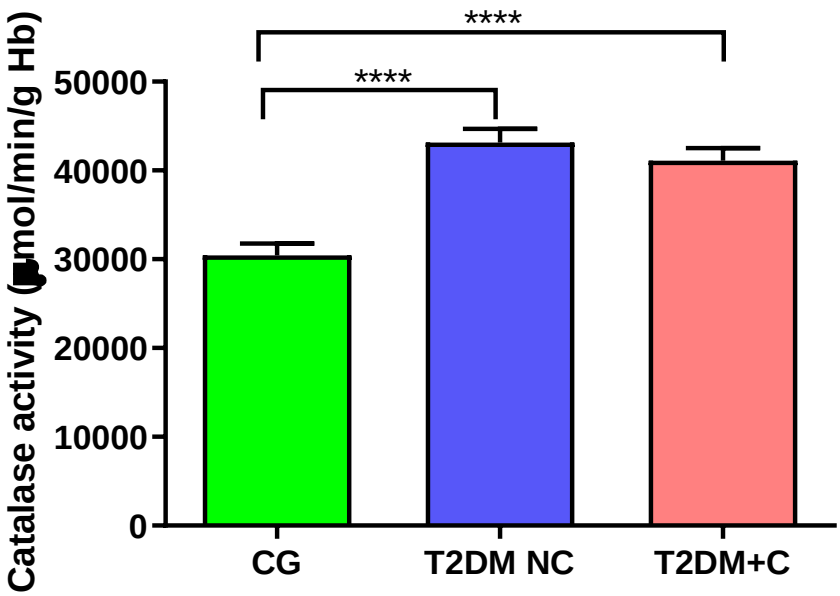

Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: G6PD - glucose-6-phosphate dehydrogenase, μmol/min/g Hb. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs *p < 0,05.

Figure 23 - The level of activity of the antioxidant enzyme G6PD in the blood of patients with type 2 diabetes mellitus and normal subjects

According to the clinical manifestations, deficiency of G6PD causes hemolysis due to medication, hemolysis due to diabetes, and hemolytic anemia due to infectious hemolysis. During oxidative stress, NADP shortage leads to insufficient reduction of oxidized glutathione when glucose-6-phosphate dehydrogenase (G6PD) activity is low enough. As a result, intracellular proteins (Heinz bodies) oxidize and accumulate, producing stiff red blood cells that are easily prone to hemolysis.

As for the indicators of catalase in erythrocytes, a significant increase in catalase activity was observed in patients with type 2 diabetes mellitus without complications 43148,26 ±7330,39 and in the group with complications 41091,35±6853,66 compared with the control 30420,59±5585,62 (p<0,0001), (Figure 24). The reason for

the increase in catalase activity is that in these patients, the glutathione cycle is first disrupted, then SOD and catalase uses its activity against oxidative stress.




Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis:CAT – catalase, µmol/min/g Hb. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs ****p < 0,0001.

Figure 24 - Activity of antioxidant enzyme catalase in blood parameters of normal subjects and patients with type 2 diabetes mellitus

Regarding the glutathione cycle, a significant Pearson correlation analysis revealed significance between some parameters when comparing oxidative stress markers and biochemical variables in all participants (Table 6). It is worth highlighting the significant positive correlations between AOPP and analytical parameters (glucose levels r= 0,283, p=0,012, HbA1c r= 0,236, p=0,038 and TG r= 0,506, p=0,000), such as between CAT and HbA1c (r= 0,261, p=0,039), CAT activity and the HOMA-IR index (r=0,262, p=0,047), GSSG/GSH ratio and TG (r=0,338 p=0,006).

In the same way, negative correlations were found between GRd and glucose (r=-0,348, p=0,008), GRd and HbA1c (r=-0,358, p=0,007), SOD and glucose (r=-0,283, p=0,024), SOD and HbA1c (r=-0,396, p=-0,001), and SOD and TG (r=-0,289, p=0,021).

Table 6 - Summary of significant (p<0.05) Pearson correlations between oxidative stress markers and biochemical variables for all participants.

| | Glucose | | HbAc1 | | HOMA-IR | | TG | | Urea | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **r** | **p** | **r** | **p** | **r** | **p** | **r** | **p** | **r** | **p** |
| **GSSG/ GSH** | 0,240 | 0,054 | 0,295 | 0,244 | 0,244 | 0,056 | **0,338** | **0,006** | 0,142 | 0,258 |
| **GRd** | **-0,348** | **0,008** | **-0,358** | **0,007** | -0,227 | 0,102 | -0,216 | 0,110 | -0,024 | 0,858 |
| **Catalasa** | 0,200 | 0,115 | **0,261** | **0,039** | **0,262** | **0,047** | 0,207 | 0,104 | -0,048 | 0,707 |
| **SOD** | **-0,283** | **0,024** | **-0,396** | **-0,001** | -0,163 | 0,212 | **-0,289** | **0,021** | -0,173 | 0,175 |
| **G-6-PD** | -0,219 | 0,102 | **-0,408** | **0,002** | 0,029 | 0,835 | **-0,270** | **0,042** | **-0,261** | **0,050** |
| **AOPP** | **0,283** | **0,012** | **0,236** | **0,038** | 0,226 | 0,053 | **0,506** | **0,000** | 0,204 | 0,073 |
| **LAT** | 0,174 | 0,162 | 0,176 | 0,157 | 0,126 | 0,333 | 0,031 | 0,805 | 0,110 | 0,378 |

In italics, statistical significance.

Moreover, negative correlations were found between G6PD and analytical parameters (HbA1c (r=-0,408, p=0,002), TG (r=-0,270, p=0,042), and urea (r=-0,261, p=0,050) (see Table 6). * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

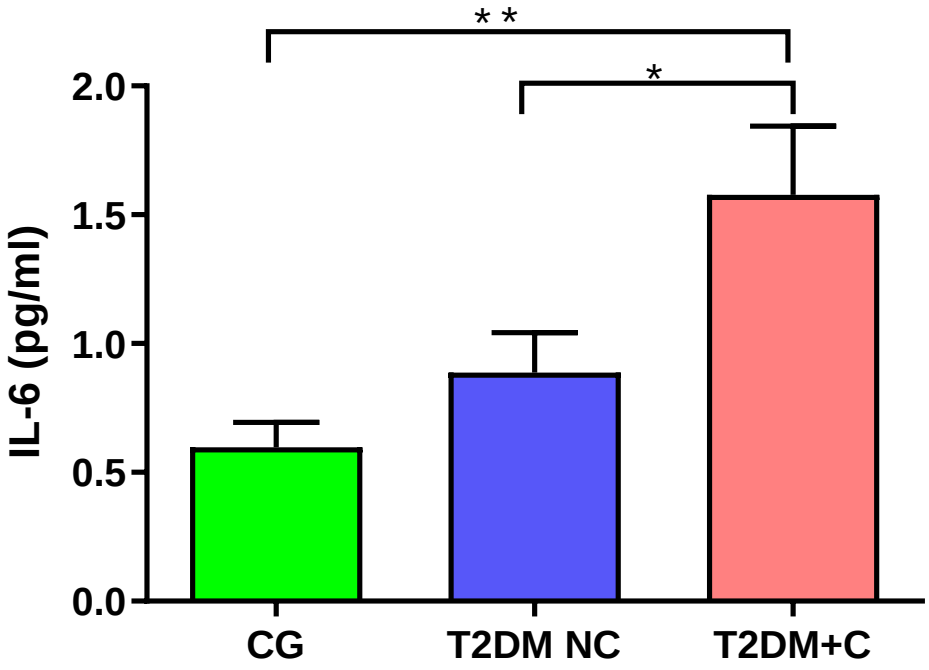
These correlations lost their significance when analyzed only in the diabetic groups. It is worth noting that the correlation between GPx activity and glucose levels was close to being significant (r = 0.359, p = 0.06).

### 3.4 To determine the spectrum of inflammatory markers (IL-6, IL-10, IL-18, and TNF-α) in the plasma of patients with type 2 diabetes mellitus and to analyze the correlation between inflammation markers, redox status and relative microRNA expression levels

To determine the role of the immune system in the development of diabetes mellitus, patients with diabetes mellitus underwent an immunological examination; in particular, indicators of cytokine immunity were studied.

To determine the state of cytokines, we determined the composition of the level of cytokines with multidirectional effects in the blood plasma of a group of T2DM patients, without vascular complications and with complications, and in the control group: pro-inflammatory cytokines - Biomarkers IL-6, IL-18, TNF-α, and anti-inflammatory cytokine - IL-10 were analyzed.

IL-6 levels were significantly higher in patients with T2DM with complications compared to the control group (p<0,01). Furthermore, an increased amount of IL-6 was detected in the diabetic complications group as opposed to the diabetes non-complications group (p <0.05). However, there was no significant difference between the group of diabetic patients without complications compared to the healthy control group (Figure 25).

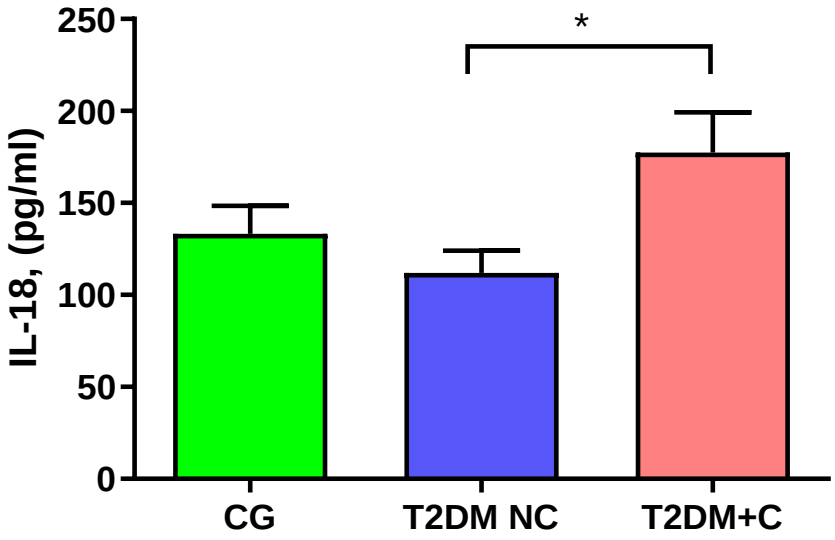



Abscissa axis: CG- controls group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: IL-6 - interleukin-6, pg/ml. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs *p<0,05; **p < 0,016.

Figure 25 - The concentration of the pro-inflammatory cytokine IL-6 in the blood plasma of patients with type 2 diabetes mellitus and normal subjects

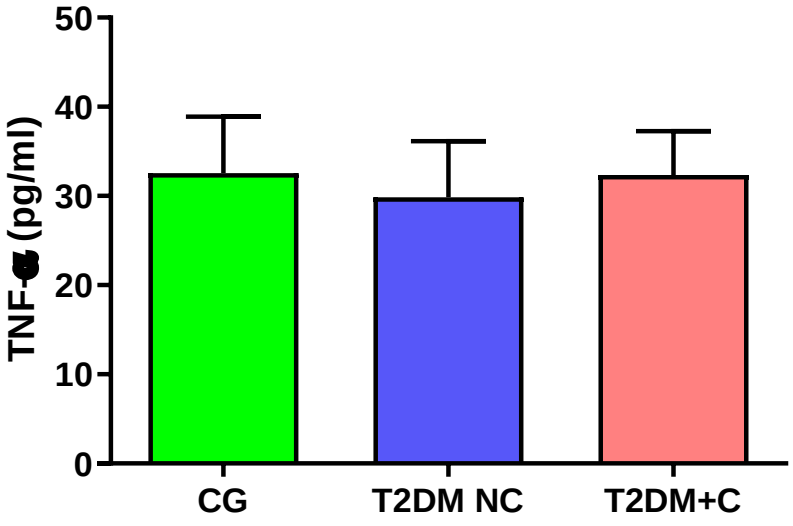
Various pathophysiological processes occur with complications of diabetes mellitus that lead to an increase in the level of interleukin 6 (IL-6) in the body. One of the main mechanisms of IL-6 elevation in diabetic complications is related to inflammatory processes that occur in body tissues during hyperglycemia (high blood glucose level). Hyperglycemia stimulates the production of pro-inflammatory cytokines such as IL-6 in response to tissue damage. Inflammation is a chronic process, so it can contribute to the development of diabetes complications. Additionally, elevated levels of IL-6 may be associated with insulin resistance, which might be a major cause of diabetes. Stimulation of the production of C-reactive protein IL-6, in turn, leads to the development of insulin resistance in the body's cells. Some studies also suggest that elevated IL-6 levels may be associated with an increased risk of diabetes complications such as diabetic retinopathy, nephropathy, or neuropathy. IL-6 has been shown to contribute to damage to the endothelium of blood vessels and increase the permeability of the vessel wall, leading to the development of microangiopathy and other complications of diabetes [197].

In addition, the level of IL-18 was significantly higher (p<0,05) in the T2DM group with complications compared to the group of T2DM patients without complications, and no difference was observed between the control group and the two diabetic groups (Figure 26).



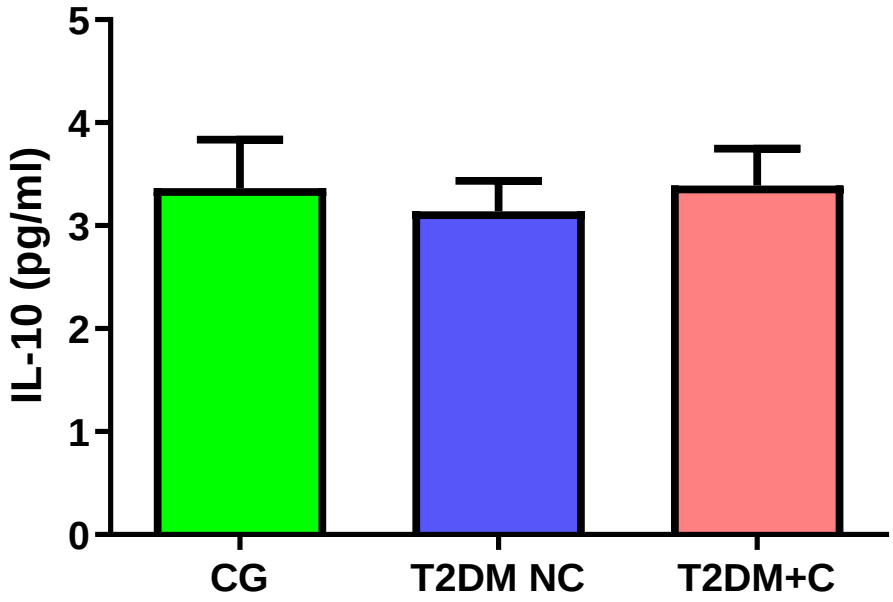

Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: IL-18 - interleukin-18, pg/ml. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs *p < 0,05.

Figure 26 - The concentration of the pro-inflammatory cytokine IL-18 in the blood plasma of patients with type 2 diabetes mellitus and normal subjects




Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: TNF-α - Tumor necrosis factorα, pg/ml. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs.

Figure 27 - The concentration of the pro-inflammatory cytokine IL-10 in the blood plasma of patients with type 2 diabetes mellitus and normal subjects




Abscissa axis: CG- control group, T2DM NC - experimental group of patients with type 2 diabetes without complications, T2DM+C - experimental group of patients with type 2 diabetes with complications. Ordinate axis: IL-10 - interleukin-10, pg/ml. Data are presented as means ± standard error of the mean (SEM). Comparisons between groups are indicated in the graphs.

Figure 28 - The concentration of the anti-inflammatory cytokine IL-10 in the blood plasma of patients with type 2 diabetes mellitus and normal subjects

The cytokine IL-10 belongs to the IL-10 family along with IL-19, IL-20, IL-22, IL-24 and IL-26. Interleukin 10 (IL-10) is a cytokine with pronounced anti-inflammatory properties, which reduces inflammation in the body and has an immunoregulatory effect. Although the level of IL-10 may increase in patients with type 2 diabetes, the concentration of this cytokine may not change with the progression of this disease, as shown in the study. One of the possible reasons for this is that in the complications of type 2 diabetes, tissue inflammation increases, and many other cytokines (e.g. IL-1β, IL-6, TNF-α) dominate the pathogenesis of the disease. These pro-inflammatory cytokines may down-regulate IL-10 expression and interfere with its anti-inflammatory action. In addition, the mechanisms of IL-10 level regulation in the body are a very complex process; that is, they may be directly related to the presence of inflammatory cells, other cytokines, and many other factors. This explains that the concentration of IL-10 does not always correspond to the severity of the disease and the presence of complications [198].

It also can inhibit the secretion of pro-inflammatory cytokines, namely IL-1. However, there were no significant differences in the concentration level of anti-inflammatory IL-10 cytokine and pre-inflammatory TNF-α concentration in blood plasma between the group of patients with and without complications of diabetes mellitus compared to the control group (Figure. 27, 28).

Pearson's significant correlation analysis showed a significant positive correlation between LAT and IL-6 ($r = 0{,}338$; $p = 0{,}009$) when comparing markers of

oxidative stress and indicators of inflammatory processes. These correlations lost their significance when analyzed only in the diabetic groups. It is worth noting that the correlation between GPx activity and glucose levels was close to being significant (r = 0.359, p = 0.06). However, there were significant correlations between: GPx and IL-10 (r = -0,358; p = 0,037); GPx and TNF-/IL-10 (r = 0.461, p = 0.008); and AOPP and TNF-/IL-10 (r = 0.393, p = 0.009). In addition, there was an approximating correlation between the years since diagnosis and G6PD (Table 7).

Table 7 - Summary of significant (p<0.05) Pearson correlations between oxidative stress markers and cytokines for all participants.

| Cytokines / Redox markers | IL-6 | |
|---|---|---|
| | r | P |
| **GSSG/GSH** | 0,253 | 0,053 |
| GRd | -0,233 | 0,100 |
| CAT | 0,074 | 0,584 |
| SOD | -0,189 | 0,158 |
| **G-6-PD** | **-0,182** | **0,196** |
| **AOPP** | 0,093 | 0,452 |
| **LAT** | **0,338** | **0,009** |

In italics, statistical significance.

Also, we analyzed the correlations between the expressions of miRNAs and oxidative and inflammatory marker status in all participants and the diabetic group only, adjusting for age, sex, and BMI in both cases (Table 4). All participants had a negative correlation between miR-21 and SOD (r = - 0,325; p = 0,001) and a positive correlation between miR-21 and GPx (r = 0,443; p = 0,001). The latter was maintained in diabetic groups (r = 0,412; p = 0,014). In addition, significant negative correlations between miR-21 and IL-10 (r = -0,453; p = 0,020) and miR-126 and IL-6 (r = - 0,466; p = 0,016) were found in diabetic patients, adjusting for sex, age, and BMI. Lastly, the positive correlation between hsa-miR-21-5p and GPx (r = 0.412; p = 0.014) was maintained in the diabetic groups.

Table 8 - Summary of significant (p < 0.05) Pearson correlations (r) between plasma miRNA levels and oxidative stress and inflammatory measurements in all participants

| miRNA | Oxidative Stress Parameters | r-value | p-value |
|---|---|---|---|
| All participants | | | |
| hsa-miR-21-5p | SOD | -0,325 | 0,001 |
| | GPx | 0,443 | 0,001 |
| | AOPP | 0,342 | 0,019 |
| hsa-miR-126-5p | SOD | 0,282 | 0,030 |

| | | | |
|---|---|---|---|
| | CAT | -0,359 | 0,007 |
| | LPO | -0,287 | 0,027 |
| All participants adjusted for sex, age, and BMI. | | | |
| hsa-miR-21-5p | GPx | 0,558 | <0,0001 |
| hsa-miR-126-5p | IL-6 | -0,480 | 0,004 |
| Diabetic patients | | | |
| hsa-miR-21-5p | GPx | 0,412 | 0,014 |
| hsa-miR-126-5p | GPx | 0,360 | 0,039 |
| | GSSG/GSH | 0,320 | 0,041 |
| Diabetic patients adjusted for sex, age, and BMI. | | | |
| hsa-miR-21-5p | GPx | 0,419 | 0,047 |
| | IL-10 | -0,453 | 0,020 |
| hsa-miR-126-5p | IL-6 | -0,466 | 0,016 |
| | GSSG/GSH | 0,493 | 0,017 |

The positive correlations between miR-126 and GPx (r =0,360; p = 0,041), and miR-126 and the GSSG/GSH ratio (r = 0,320; p = 0,041) were found in diabetic patients; the latter was maintained in diabetic patients, adjusting for sex, age, and BMI (r = 0.493, p = 0.017) (Table 8).

### 3.5. To study the diagnostic value of markers associated with the presence of type 2 diabetes mellitus and its vascular complications in the study groups

The ROC curve graph was used in the study to evaluate the possibility of using microRNA as a diagnostic marker to distinguish disease cases from normal cases. It is assessed by analyzing the receiver operating characteristic (ROC) curve. Area under the ROC (AUC) curve (AUCROC - area under the receiver operating characteristics) - this indicator is designed to measure how well two diagnostic groups (disease and normal state) can be distinguished.

To investigate the diagnostic accuracy of circulating miR-21 and miR-126, as well as oxidative stress and inflammatory markers, in distinguishing type 2 diabetics from healthy individuals, receiver operating characteristic (ROC) curves were constructed. In the study, the area under the curve (AUC) and the 95% confidence interval were monitored for the markers under analysis in all participants (Table 9). It should be noted that several markers appear as candidates for having a significant predictive value for diabetes. In the study, only the following biomarkers with a sensitivity of 50 % SI and an AUC si of more than 750 were selected: HbA1c, CAT, miR-21, GPx, AOPP, and LPO (these markers are underlined in Table 9).

Table 9 - AUC and 95% confidence intervals for studied markers distinguishing between control and T2DM groups.

| **Blood parameters** | **AUC** | **95% Confidence Interval** | | **p-value** |
|---|---|---|---|---|
| | | **Мин** | **Max** | |

| | | | | |
|---|---|---|---|---|
| HbAc1 | 0,957 | 0,902 | 1,000 | **0,000** |
| HOMA IR | 0,769 | 0,626 | 0,911 | 0,027 |
| Urea | 0,587 | 0,462 | 0,712 | 0,194 |
| hsa-miR-21-5p | 0,877 | 0,772 | 0,983 | **0,000** |
| hsa-miR-126-5p | 0,245 | 0,078 | 0,411 | 0,034 |
| GSSG/GSH | 0,721 | 0,488 | 0,953 | 0,069 |
| AOPP | 0,792 | 0,642 | 0,941 | **0,003** |
| LAT | 0,775 | 0,561 | 0,990 | **0,023** |
| CAT | 0,913 | 0,830 | 1,000 | **0,000** |
| GRd | 0,249 | 0,104 | 0,394 | 0,004 |
| G-6-PD | 0,277 | 0,147 | 0,408 | 0,010 |
| SOD | 0,125 | 0,036 | 0,214 | 0,000 |
| GPx | 0,796 | 0,549 | 0,897 | **0,023** |
| IL-6 | 0,778 | 0,470 | 0,811 | 0,150 |
| IL-10 | 0,487 | 0,209 | 0,766 | 0,917 |
| IL-18 | 0,597 | 0,353 | 0,840 | 0,426 |
| TNF-α | 0,557 | 0,303 | 0,811 | 0,640 |

However, when ROC analysis was completed within the T2DM NC and T2DM+C groups to assess the predictive power of markers for developing vascular complications, these differences disappeared, except for GPx and IL-6 (Table 10).

Table 10 - AUC and 95% confidence intervals for studied markers distinguishing between T2DM NC and T2DM+C.

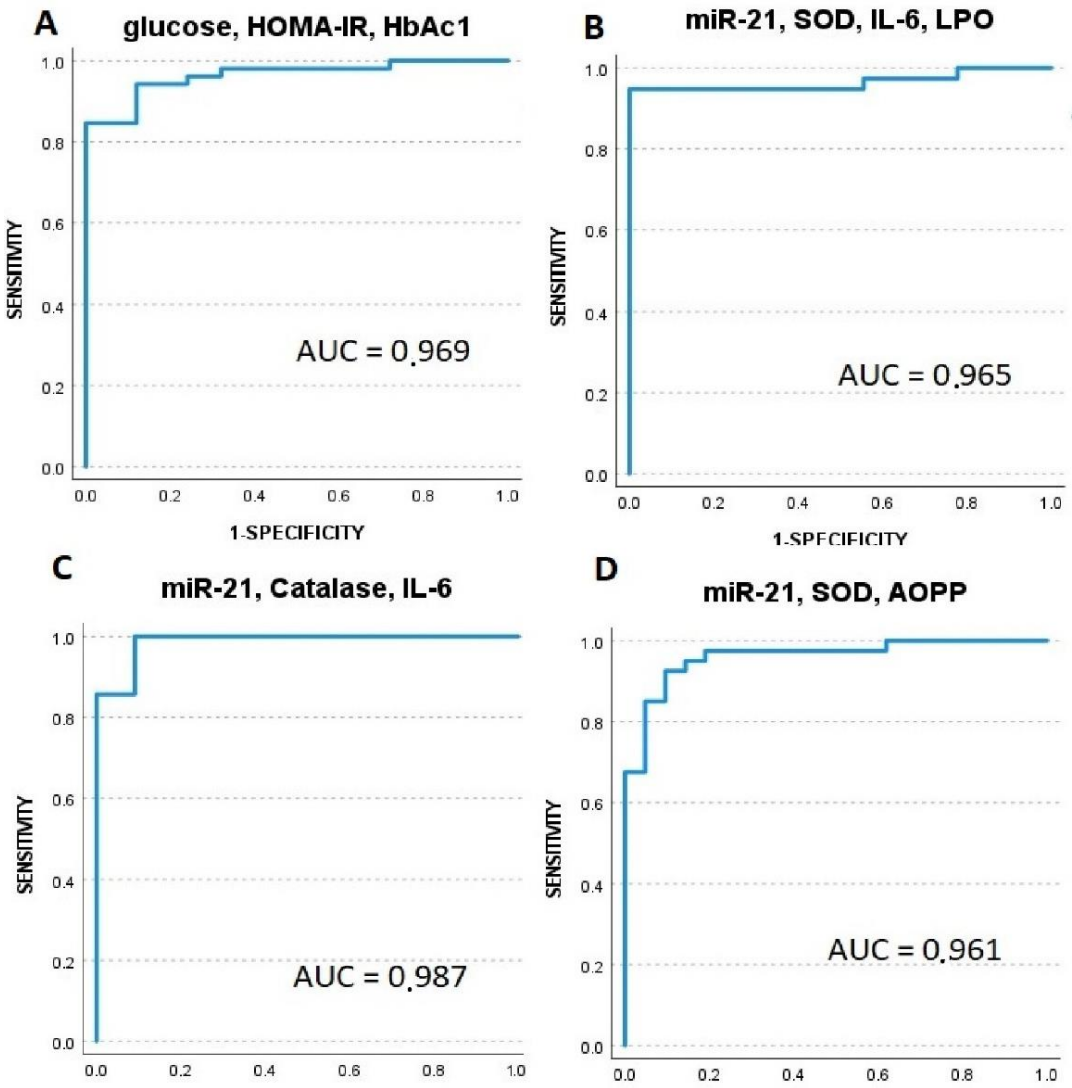
| **Blood parameters** | **AUC** | **95% Confidence Interval** | | **p-value** |
|---|---|---|---|---|
| | | **Min** | **Max** | |
| HbA1c | 0,633 | 0,390 | 0,875 | 0,281 |
| Urea | 0,578 | 0,374 | 0,853 | 0,332 |
| hsa-miR-21-5p | 0,520 | 0,355 | 0,685 | 0,810 |
| hsa-miR-126-5p | 0,325 | 0,133 | 0,517 | 0,101 |
| CAT | 0,371 | 0,131 | 0,612 | 0,295 |
| IL-6 | 0,708 | 0,490 | 0,927 | 0,040 |
| IL-18 | 0,720 | 0,507 | 0,932 | 0,074 |
| AOPP | 0,511 | 0,261 | 0,762 | 0,926 |
| GPx | 0,796 | 0,549 | 0,897 | 0,023 |
| G-6-PD | 0,402 | 0,200 | 0,604 | 0,357 |
| SOD | 0,650 | 0,443 | 0,856 | 0,161 |

Logistic regression models compared classical diagnostic parameters for diabetes diagnosis with biochemical and plasmatic biomarkers. Notably, some of these models revealed the highest predictive risk for diabetes:

miR-21, SOD, and IL-6: Exp (B) = 2.235, chi2 = 49.015, $p = 0.006$;

miR-21, SOD, IL-6, and LPO: Exp (B) = 4.22, chi2 = 50.731, $p = 0.001$; and miR-21, CAT, IL-6, and BMI: Exp (B) = 3.818, chi2 = 46.107, $p = 0.001$.

We applied ROC curve analysis to evaluate the predictive values of these markers. Results demonstrated that AUC values for models with miR-21, SOD, IL-6, and LPO and miR-21, SOD, and AOPP were similar (Figure 28 B, D) or higher (models with miR-21, CAT, and IL-6) than the predictive AUC value for classical diagnostic parameters (glucose, HbA1c, and HOMA-IR) (Figure 29 A, C).




Abscissa axis: 1- specificity. Ordinate axis: sensitivity. Receiver operator characteristic (ROC) curves generated for sensitivity analysis show diagnostic performances of plasma markers, including the following models: (A)-glucose, HOMAIR, HbA1c; (B)-miR-21, SOD, IL-6, LPO; (C)-miR-21, catalase, IL-6; (D)-miR-21, SOD, and AOPP - AUC-area under the curve.

Figure 29 - Receiver operator characteristic (ROC) curves generated for sensitivity analysis

In this table, the diagnostic value of various indicators studied about the risk of developing complications in all patients was calculated by binary logistic regression

analysis, and the predictive values of the development of vascular diabetic complications in all participants were presented.

We performed binary logistic regression analysis to analyze the diagnostic value of the different parameters for predicting the risk of developing complications. Binary logistic regression analysis is an analysis that shows how reliable a biomarker is.

When we compared other models of classic clinical parameters, such as glucose, HbA1c, years since diagnosis, glucose, and urea, only the following models had a significant value, although with lower specificity: miR-126 and IL-6; miR-126 and years since diagnosis; miR-21 and IL-6; and miR-21, IL-6, AOPP (Table 11). Moreover, among the identified models, the miR-126 and years since diagnosis model has the most significant predictive power (p = 0.012). The advantage of the miR-126 and years since diagnosis model is that it requires less resources to implement and analysis to identify only one epigenetic marker.

Nevertheless, according to binary logistic regression analysis, these new biomarkers for diabetics in three suggested combinations could be utilized as valid predictors of vascular diabetic complications.

Previously, miR-126-5p and miR-21-5p were used as biomarkers for breast cancer and other diseases. The identified association between the levels of miR-126-5p and miR-21-5p and the presence of diabetes complications may indicate that metabolic pathways that are also involved in the development of breast cancer may be involved in the development of vascular pathology due to diabetes, which may contribute to understanding the mechanisms of pathogenesis of these pathologies.

Table 11 - represents the predictive values for developing vascular diabetic complications among all participants, calculated by binary logistic regression analysis.

| | AUC, (95%) | Exp(B)=OR | $Chi^2$ | p-value |
|---|---|---|---|---|
| Glucose, years since Dx, HbA1c | 0,860 (0,782-0,937) | 0,464 | 33,192 | 0,001 |
| Glucose, years since Dx, urea | 0,888 (0,820-0,956) | 0,464 | 38,394 | 0,001 |
| hsa-miR-126-5p, ИЛ-6 | 0,793 (0,673-0,913) | 0,579 | 18,880 | 0,041 |
| hsa-miR-126-5p, years since Dx | 0,868 (0,785-0,950) | 0,523 | 30,655 | 0,012 |
| hsa-miR-21-5p, IL-6 | 0,810 (0,701-0,920) | 0,600 | 17,879 | 0,048 |
| hsa-miR-21-5p, IL-6, AOPP | 0,801 (0,687-0,915) | 0,600 | 19,522 | 0,048 |

We also assessed the diagnostic performance of different models in discriminating the T2DM NC and T2DM+C phenotypes. However, binary logistic regression analysis revealed that only IL-6 had a significant predictive value for developing vascular complications in the diabetic groups: Exp (B) = 2,347, OR: 1,136–4,848, p = 0,021.

## 4 - DISCUSSION OF RESEARCH FINDINGS

Modern molecular biomarkers are not sensitive enough to predict the onset and course of diseases caused by diabetes. The analysis of epigenetic mechanisms, in particular, the expression of microRNA, allows to expand the scale of this research, creating new methods of diagnosis and personalized treatment of patients. Also, microRNA expression plays an important role in reducing oxidative stress and diabetic complications.

As a chronic disease, diabetes is caused by microvascular and macrovascular damage. It increases the risk of several other diseases and adversely affects several organs, such as the brain, kidney, heart, and eye. The life expectancy and quality of life of patients with type 2 diabetes often depend on the development of "late" chronic complications, and the intensity of their development directly depends on the quality of DM compensation [199].

Type 2 diabetes mellitus is associated with oxidative stress and low-grade inflammation, resulting in endothelial dysfunction (ED). Clinical studies demonstrate that tight glycemic control does not significantly reduce the appearance of diabetic vascular complications [39]. On the other hand, increased oxidative stress and inflammation can lead to insulin resistance and impair insulin secretion. Additionally, changes in epigenetic control can influence these processes [38]. There is a need to analyze changes in inflammatory and oxidative state markers and miRNA expression to find biological markers that have sufficiently high predictive power for developing vascular complications in diabetic patients.

Fasting blood glucose is the current diagnostic criterion for diabetes, being globally accepted for almost a decade. However, information regarding insulin resistance and type 2 diabetes has increased exponentially during the last few years, suggesting the existence of complex networks that regulate energy balance in close association with the immune system and chronic inflammation [200]. A broader view of this paradigm prompted us to research new potential biomarkers, such as circulating miR-126-3p and miR-21-5p, since these miRNAs were associated with endothelial dysfunction and inflammation, two milestones of T2DM progression.

In this study, we found significantly increased c-miR-21 and decreased c-miR-126 plasma levels in diabetes patients with and without complications, compared with healthy controls, in the Mexican population (Figure 12, 13), even after adjusting for sex and age (data not shown). These results are consistent with several published studies. In humans, increased plasma miR-21 is considered a predictor of later development of T2DM in prediabetic individuals [201]. Studies have reported that miR-21 exerts its deleterious actions in diabetic retinopathy [202], diabetic nephropathy [203], beta-cell apoptosis [204], and is associated with pancreatic islet inflammation and insulin resistance initiation [205]. An in vitro study showed that high glucose levels increased miR-21 expression [206]. This study revealed a positive correlation between c-miR-21 and glycemic impairment (plasmatic glucose and HbA1c) in diabetic patients. Additionally, miR-21 is considered a pro-inflammatory miRNA induced by several pro-inflammatory molecules; increasing miR-21 leads to promoting the NF-κB pathway and

subsequent NLRP3 inflammasome activation, acting directly on Toll-like receptors and by targeting A20 [204, 207].

As a result, it is reasonable to expect increased miR-21 to lead to higher production of pro-inflammatory ILs. functional in vitro experiments with mimic miR-21 elevated the levels of IL-6 and IL-1β and reduced IL-10 [208]. In this study, we did not find a relation between miR-21 expression and pro-inflammatory ILs; nevertheless, we found a negative correlation between miR-21 and anti-inflammatory IL-10 in diabetic patients after adjusting for sex, age, and BMI (Table 8). Our results are similar to those of a recent publication that showed that miR-21 inhibition in myeloid cells in a sepsis model was associated with increased anti-inflammatory IL-10 in vivo and in vitro [199,104].

Otherwise, c-miR-126 showed lower levels in T2DM NC and T2DM+C than controls. These results are consistent with previous studies that reported reduced c-miR-126 levels as a biomarker for pre-diabetes and T2DM [144, 118, 209]. Studies have demonstrated that miR-126 decreases VCAM-1 expression and can promote insulin resistance by inhibiting IRS-1 [116, 210]. It is involved in regulating vascular integrity and angiogenesis and is one of the most expressed miRNAs in the endothelium [211]. Animal model studies have revealed that reduced expression of miR-126 is significantly associated with T2DM and brain damage. On the contrary, high expression of miR-126 is associated with a lower activity of NLRP3, reducing inflammation related to retinopathies and inducing brain damage in the mouse diabetic model [120, 212]. The current study also correlated decreased plasma miR-126 with high blood glucose and HbA1c (Figure 14). This correlation suggests that worsened hyperglycemia parameters might result in the reduced delivery of miR-126 to the circulatory system, contributing to endothelial dysfunction.

The expression of hsa-miR-126-5p was decreased in both groups of patients, but it was not found to be significant as a predictive value. Given that hsa-miR-21-5p has anti-inflammatory effects due to its ability to stimulate the activation of NF-kB and NLRP3 pathways, it is involved in recognizing antioxidant pathways and ROS homeostasis. According to these preliminary results, circulating microRNAs and traditional markers play an important role in assessing disease progression. ROC analysis proved that hsa-miR-21-5p has the same predictive value as HbA1c in diagnosing T2DM.

The notable contribution of this work is a complete study of the redox status in T2DM patients in peripheral blood samples, as well as an analysis of how the antioxidant system responds to oxidative stress in diabetes patients with and without VCs. First, we found that patients, regardless of complications, had elevated levels of AOPP and LPO compared with controls. AOPP and LPO are products of reactions involving ROS with protein side chains and lipids [212–214]. High plasma AOPP concentrations were related to atherosclerotic lesions and vascular inflammation in diabetic patients [212, 215]. Moreover, AOPP levels were proposed as an independent risk factor for endothelial dysfunction in individuals in the early stages of diabetes without albuminuria [212]. Recent animal model and in vitro studies demonstrated that AOPPs are not merely OE products but also

promoters of redox-sensitive inflammation in the vasculature, mitochondrial dysfunction, and oxidative stress, causing early diabetic nephropathy [214-216]. These findings, in part, were confirmed in our study when we found significant positive associations between AOPP levels and glycemic impairments (plasmatic glucose and HbA1c) and TG. Moreover, the AUC of AOPP exhibited high values in diabetes patients compared with controls (Table 9), which may make this marker a good predictor for diabetes. In addition, including this marker in a stepwise logistic regression with miR-21 and IL-6 revealed a significantly high association with the development of vascular complications in diabetic groups (Table 11).

Concerning the antioxidant defense of diabetic patients, we found a significant reduction in erythrocyte SOD and GRd activities and a significant increase in CAT activity in diabetic patients, regardless of VCs, compared with controls. In the same way, the T2DM+C group showed decreased G6PD activity versus the control group.

Superoxide dismutase is a primary defense enzyme because it decomposes toxic superoxide anions to produce $H_2O_2$, which is then converted to non-toxic $H_2O$ and $O_2$ by GPx and CAT. We measured cytoplasmatic SOD, which has Cu/Zn in its active center and is the most sensitive to oxidative stress and high glucose levels. Approximately 50% of the decrease in activity of SOD could be explained by the enzymatic glycosylation of its center of enzymatic activity under hyperglycemic conditions [217]. Based on these data, it is altogether feasible that we found significant negative correlations between SOD and glucose levels, HbA1c, and TG in this study (see Table 6). Reports about SOD activity in diabetes are controversial, as some researchers reported no change in SOD activity, whereas others found an increase [218]. Our results align with those observed in pre-diabetics compared with control [219] and in T2DM patients versus control [220]. Studies in animal diabetic models also detected decreased SOD activity [221].

The increase in catalase activity observed in this study suggests an adaptive response that may be associated with increased H2O2 production as a result of inactivity of the SOD enzyme.

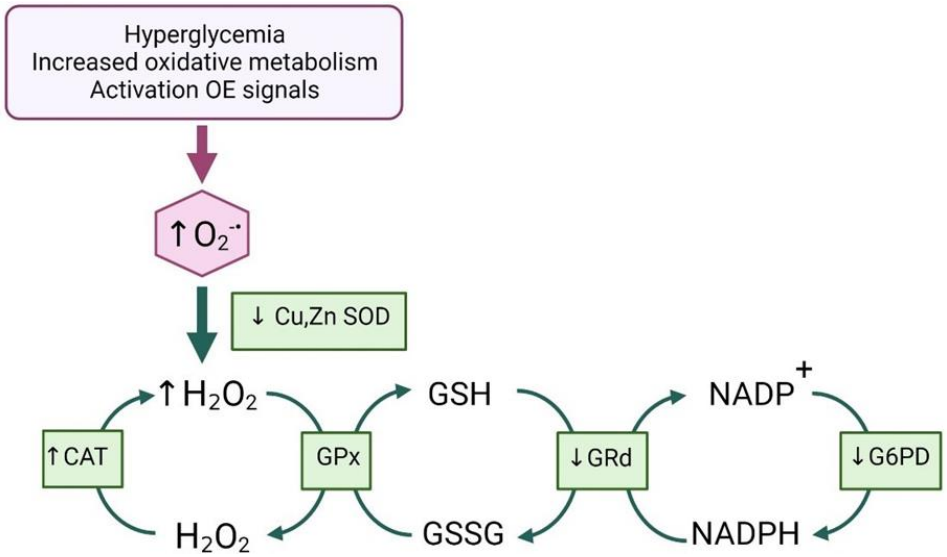
Catalase is predominantly responsible for catalyzing the conversion of $H_2O_2$ into $H_2O$ and $O_2$ in human erythrocytes [222]. Although a decrease in CAT activity was reported in some studies in patients with nephropathy [223,224], in others, its increased activity may mean a more significant effort made by the erythrocytes to counteract the accumulation of H2O2 as a result of SOD inactivity [225]. At the same time, a decrease in the expression level of miR-126 leads to an increase in the activity level of the catalase enzyme, according to the source of bioinformatics data (targets can). According to bioinformatic data, miR-126 directly interacts with the catalase gene. A positive correlation between catalase activity and HbA1c and HOMA (Table 6) should be noted. Thus, the ROC curve analysis suggested that CAT activity changes give it high predictive power for diabetes (Table 9).

The second enzyme in erythrocytes responsible for the disposal of $H_2O_2$ is glutathione peroxidase (GPx) [226]. This enzyme is part of the glutathione cycle, using the electrons and protons that GSH releases when converted to GSSG to reduce ROS. GPx responses in T2DM were controversial in different

publications. GPx was increased in patients with uncontrolled T2DM compared with pre-diabetics [217, 218] and decreased compared with controls [227, 228]. With our data in mind, we suggest that GPx activity may increase in conditions of excessive $H_2O_2$ production as an adaptive response against the excess hydrogen peroxide. Gunawardena et al. reported higher GPx activity in T2DM patients with poor glycemic control that was in parallel with the MDA levels [217]. In the present study, GPx activity tended to increase, but this increase was not significant, probably due to a small sample size. Moreover, GPx activity was significantly and positively correlated with TNF-α/IL-10 and increased miR-21 expression, the latter in both diabetic and nondiabetic groups adjusted for sex, age, and BMI (Table 8). Notably, the AUC of GPx exhibited high and significant values not only when at risk for developing diabetes but also in developing vascular damage in the diabetic group (see Tables 9 and 10).

A decreased GSH may depend on reducing GRd activity or competition for NADPH between GRd and G6PD in the polyol pathway. The latter explanation makes sense because diabetic patients show a positive correlation between GSH and G6PD ($r = 0{,}312$, $p = 0{,}031$). Several patients and animal model studies detected decreased GSH, GRd, and G6PD levels [225,229–231]. As a result, an increased GSSG/GSH ratio had a significant positive correlation with TG and an almost substantial positive correlation with glucose, supporting the idea that this oxidative stress marker is related to poor metabolic conditions.

The exact mechanisms that affect glutathione metabolism in patients with diabetic complications are unclear, and further studies are needed to evaluate the observed problems. However, our study provides complete information on the redox status in the blood of Mexican diabetics (see Figure 30)




Significant reduction of glucose-sensitive SOD activity causes an increase in CAT compensative activity, and GPx activity tends to increase. Decreased G6PD activity limits NADPH levels, contributing to the reduction of GRd activity, which leads to an increased GSSG/GSH ratio. All arrows indicate the directions of biochemical reactions. Created with BioRender.com, (accessed on 16 August 2022).

Figure 30 - Summary of redox status changes detected in the population studied.

This study revealed significantly increased IL-6 and IL-18 levels in the T2DM + C group concerning the T2DM NC group, as well as compared with the control group in the case of IL-6. Experimental animal studies demonstrated that activation of the NF-κB transcription factor and the NLRP3 inflammasome are involved in a chronic inflammatory response to high oxidative stress in T2DM, which leads to an increase in IL-1β and IL-18, playing a crucial role in the development of diabetes [232], aggravating pro-coagulant activity. Recent studies correlated the overexpression of pro-inflammatory (IL-1β, IL-6, TNF-α, and VEGF) and profibrotic genes (ICAM-1 and VCAM-1) with the appearance of nephropathies in diabetic patients [233]. In our study, IL-6 showed a high predictive value for VC development, which was demonstrated using ROC curves and binary logistic regression analysis. However, IL-6 is highly associated with overweightness and obesity, both states that are related to chronic low-grade inflammation. In this study, the three groups of participants had no significant differences in BMI, weight, or waist circumference. This makes us think that the increase in IL-6 is due not only to weight gain but also to the worsening inflammatory state caused by the disease. Binary logistic regression analysis revealed that models including IL-6 and miR-21, and AOPP had significant predictive value for developing VCs in the studied population.

Furthermore, although we did not find differences in IL-10 levels between groups, a significant negative correlation between miR-21 and IL-10 was detected in diabetic patients after adjusting for sex, age, and BMI (Table 8). These findings are consistent with several authors who have reported that disequilibrium between pro-inflammatory and anti-inflammatory cytokines aggravates the complications related to T2DM [234]. Studies in animal models also confirm the active participation of ILs in developing diabetic complications, such as retinopathy [235] or neuropathy [236].

This was the first systematic investigation of the relationship between plasma microRNA expression and a broad spectrum of oxidative stress parameters in the diabetic population. Notably, our correlational analysis showed an inverse link between miR-21 expression and SOD activity in all participants, corroborating LaSala´s results that demonstrated that miR-21 was associated with a prediabetic status and exhibited predictive value for diabetes detection [202, 207]. In addition, we found the enzyme CAT to be a candidate for predicting diabetes, with a high sensitivity, specificity, and AUC, similar to HbA1c. As the disease progresses, a positive correlation between miR-21 and GPx makes us think that GPx plays an essential role in evaluating the risk of suffering complications. This positive correlation is maintained even in the analysis of diabetic patients only, adjusted for sex, age, and BMI (Table 8).

We also corroborated the regulatory role of c-miRNAs in regulating the inflammatory state; in this case, increased c-miR-21 expression was correlated with decreased production of anti-inflammatory IL-10, and decreased miR-126 levels were negatively correlated with pro-inflammatory IL-6. Both correlations were detected in diabetic patient groups after adjusting for sex, age, and BMI (Table 8).

Using both ROC curve analysis and stepwise logistic regression analysis, we identified that models including miR-21, IL-6, CAT, and SOD had a high predictive value for T2DM diagnosis, similar to classical diagnostic parameters (Figure 29). Therefore, we suggest that IL-6 be considered a predictor for the development of vascular complications in the diabetic population. Additional studies are needed to evaluate the possible predictive power of miR-21, miR-126, GPx, and AOPP to develop vascular complications. In addition, LPO and SOD markers should also be considered for future studies.

A limitation of this study was the reduced sample size. Additionally, the T2DM NC group included a different number of men and women than the other two groups due to randomized recruitment. However, there were no significant differences in studied parameters between genders.

## CONCLUSION

The following conclusions were drawn from the study:

1. It was proved that circulating biochemical indicators (HbA1c, glucose, insulin, HOMA-IR index, total cholesterol, triglyceride) in the blood of patients underwent changes in comparative analysis in patients with DM2T without and with vascular complications and control group;

2. In this study, we found significantly increased c-miR-21 and decreased c-miR-126 plasma levels in diabetes patients with and without vascular complications, compared with healthy Controls in the Mexican population. The differences between diabetic and diabetic complicated groups were not found. However, we reported that glycemic impairment (plasmatic glucose and HbAc1 levels) positively correlated with pro-inflammatory miR-21 and a negative correlation with endothelial protective miR-126 in diabetic patients. These correlations suggest that worsened hyperglycemia parameters might result in the reduced delivery of miR-126 to the circulatory system, contributing to endothelial dysfunction.

3. This work's notable contribution is a complete study of the redox status in the peripheral blood samples of T2DM patients and an analysis of how the antioxidant system responds to oxidative stress in diabetes patients with and without vascular complications. During the disease, an increase in protein oxidation products (AOPP) and an increase in the level of lipid peroxidation (LPO) were detected.

As the disease progresses, increased CAT activity compensates for decreased SOD activity, and GPx activity tends to increase. Decreased GRd and G6PD activity led to an increased GSSG/GSH ratio. The positive correlations between miR-126 and GPx, and between miR-126 and the GSSG/GSH were found in diabetic patients, adjusting for sex, age, and BMI.

4. The level of the inflammatory cytokine IL-6 was significantly increased in both diabetic groups. The negative correlations between miR-21 and IL-10 and between miR-126 and IL-6 in the group of patients with T2DM, adjusted for sex, age, and BMI, indicate that these ILs make a significant contribution to disease development. IL-6 was a predictive biomarker of vascular injury in a studied population of diabetic patients.

5. The ROC curve analysis to evaluate the predictive values of studied biomarkers for diabetic diagnosis revealed that models included miR-21, SOD, IL-6, and LPO had similar or higher predictive AUC values than classical diagnostic parameters (glucose, HbA1c, and HOMA-IR). So, we recommended considering miR-21, SOD, LPO, and IL-6 as prognostic markers for t2dm. Finally, our data demonstrated that IL-6 exhibited predictive value for VC development in the studied population. Moreover, c-miR-21 and c-miR-126, along with GPx and AOPP levels, should be considered possible markers for VC development in future studies.

**Evaluation of the complete fulfillment of the tasks**

The objectives set in the dissertation were fully accomplished. A complete analysis of the redox state and the antioxidant defense system was carried out in the case of type 2 diabetes. Expression of circulating hsa-miR-21-5p, hsa-miR-126, and

IL-6 in microRNAs in type 2 diabetes were found to be predictive biomarkers of vascular damage in a patient population.

**Recommendations for using baselines and results**

The experience gained can be used for preliminary treatment purposes.

The information presented in the dissertation was included in lectures on the disciplines “Molecular Endocrinology”, “Molecular Membranology” at Kazakh National University named after al-Farabi. The data obtained from our research work can be used in lectures in biochemistry, molecular biology, endocrinology, and physiology.

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# Appendix A

Informed consent to participate in the study

Título del Estudio: ***Determinación de los MicroRNAs circulantes y el estado Redox como biomarcadores y predictores de la gravedad de Diabetes Mellitus Tipo II en pacientes adultos del estado de Jalisco, México.***

**HOJA DE INFORMACION AL PACIENTE O PARTICIPANTE**

**Objetivos**.

La Universidad de Guadalajara, Mexico, y la Universidad de Granada, España, unen sus esfuerzos para realizar un estudio sobre la diabetes mellitus tipo II (T2DM). Esta enfermedad afecta a un gran número de personas en todo el mundo. Durante varios años la persona puede permanecer sin síntomas, mientras la enfermedad está produciendo daños significativos en el organismo, afectando a varios órganos. Enfermedad cardiovascular es la complicación de mayor prevalencia relacionada con la diabetes y alrededor del 75% de los pacientes diabéticos mueren debido a las consecuencias cardiovasculares incluyendo la afectación de las arterias coronarias. El propósito general del estudio es analizar las moleculas que podrían servir como posibles marcadores de la T2DM y predictores de las complicaciónes relacionadas con la salud cardiovascular.

Los microRNAs son reconocidos como una nueva clase de biomarcadores que tienen una alta especificidad para el estado de la enfermedad y pueden considerarse unos buenos indicadores de patogénesis y progresión de la enfermedad. En este estudio se pretende analizar la expresión de los miRNAs relacionados con el metabolismo de glucosa y la disfunción endotelial en pacientes mejicanos diagnosticados con DMT2 al menos 5 años previos a la extracción de sangre. Además, se evaluará el estado oxidativo e inflamatorio de los participantes, y se hará la correlación entre el estado de su salud cardiovascular y los parámetros analizados.

**Metodología empleada**

Si usted acepta participar en el estudio, ocurrirá lo siguiente:

Usted será citado a un centro hospitalario, indicado por el personal especializado. Deberá acudir en ayunas. El personal cualificado del laboratoio clínico le sacará una muestra de sangre de aproximadamente 8 ml, utilizando tubos EDTA-$Na_2$. La sangre se guardará a 4ºC y se utilizará para los análisis descritos en este estudio, con fines de investigación.

Se le preguntará sobre el tiempo que lleva después de que fui diagnosticado de diabetes tipo II, y si tiene alguna complicación de tipo cardiovascular: tensión arterial alta, insuficiencia cardiaca, arritmia diagnosticada, cardiopatía coronaria, accidente cerebrovascular. Si toma de forma habitual algún tipo de medicamento prescrito.

Su participación no tiene ningun tipo de riesgo para la salud.

A cambio, los **beneficios** esperados son:

**CONSENTIMIENTO POR ESCRITO DEL REPRESENTANTE**

Título del estudio: Determinación de los MicroRNAs circulantes y el estado Redox como biomarcadores y predictores de la gravedad de Diabetes Mellitus Tipo II en pacientes adultos del estado de Jalisco, México.

Yo, (nombre y apellidos) ....., con D.N.I. nº……

**en calidad de** (relación con el participante) **.....**

**de** (nombre del participante) **.....**

**He hablado con el investigador responsable del estudio.....**

**.....**

**He leído la hoja de información que se me ha entregado.**

**He podido hacer preguntas sobre el estudio. /profesional responsable del estudio**

**He recibido respuestas satisfactorias a mis preguntas.**

**He recibido suficiente información sobre el estudio.**

**Comprendo que la participación es voluntaria.**

**Comprendo que puede retirarse del estudio:**

4. **Cuando quiera.**
5. **Sin tener que dar explicaciones.**
6. **Sin que esto repercuta en sus cuidados médicos.**

**Y presto mi conformidad con que** (nombre del participante) **.....**

**..... participe en este estudio.**

**Fecha** **Firma del representante**

**Fecha** **Firma del profesional responsable del estudio y D.N.I.**

**CONSENTIMIENTO POR ESCRITO DEL PACIENTE O PARTICIPANTE**

**Título del estudio:** Determinación de los MicroRNAs circulantes y el estado Redox como biomarcadores y predictores de la gravedad de Diabetes Mellitus Tipo II en pacientes adultos del estado de Jalisco, México.

**Yo,** (nombre y apellidos) **.....,** con D.N.I. nº……

**He hablado con el profesional responsable del estudio**

**…… …….**

**He leído la hoja de información que se me ha entregado.**

**He podido hacer preguntas sobre el estudio.**

**He recibido suficiente información sobre el estudio.**

**Comprendo que mi participación es voluntaria.**

**Comprendo que puedo retirarme del estudio:**

1. **Cuando quiera.**
2. **Sin tener que dar explicaciones.**
3. **Sin que esto repercuta en mis cuidados médicos.**

**Presto libremente mi conformidad para participar en el estudio.**

**Las muestras obtenidas en este estudio sólo serán utilizadas para los fines específicos del mismo.**

**Fecha** **Firma del paciente o participante**

**Fecha** **Firma del profesional responsable del estudio y D.N.I.**