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PhD THESIS

CARDIOVASCULAR AND DIGESTIVE IMPLICATIONS OF METFORMIN AND TYPE 2 DIABETES

A B S T R A C T

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T i m i s o a r a
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INTRODUCTION

Short history: Doctors began to notice the relationship between diet and diabetes in the 19th century, when type 2 diabetes appeared. The first source of information comes from the Egyptian Ebers Papyrus from 1550 BC, which describes symptoms such as excessive thirst and frequent urination.

Around the 1920s, it was discovered that the pancreas secretes a substance called insulin, which helps regulate blood sugar levels. This discovery opened up new ways to treat diabetes with insulin. Later in the 1950s, type 2 diabetes was described as a different variant of type 1 diabetes, which occurs when the insulin-producing cells in the pancreas are completely destroyed. Type 1 diabetes has other characteristics and occurs when the insulin-producing cells in the pancreas are completely destroyed.

Problematic: Because it is associated with both cardiovascular disease and non-alcoholic fatty liver disease, type 2 diabetes is an important part of the metabolic syndrome, which compromises the function and integrity of the cardiovascular system as well as the digestive system as a result of the chronic treatment that patients receive. Insulin resistance and chronic hyperglycemia are decisive factors for cardiovascular diseases such as coronary heart disease, hypertension and stroke. Endothelial dysfunction, chronic inflammation, and functional and structural changes at the visceral level increase the risk of atherosclerosis and cardiovascular events. It is important to note that metformin can lead to uncomfortable digestive symptoms such as discomfort, nausea and diarrhea. However,

Perspectives:A better understanding of the effects of metformin and type 2 diabetes on the cardiovascular and gastrointestinal systems may help uncover potential complications. From a pharmacological point of view, we will examine the putative molecular mechanisms involved in the effect that metformin has on human hepatocytes - HepaRG, as well as in HT -29 and HCT-116, two colorectal cancer cell lines and a healthy colon cell line, CCD-841 CoN. The relationship between cardiovascular effects and different levels of liver fibrosis in patients with type 2 diabetes and non-alcoholic fatty liver disease will be clinically examined.

GENERAL PART

1. OVERVIEW OF TYPE 2 DIABETES

1.1. BRIEF HISTORY OF DIABETES

Type 2 diabetes (T2D) is a chronic medical condition characterized by the body's resistance to insulin produced by the pancreas and high blood glucose levels. This condition was first described in the 2nd century BC. in Ancient Egypt(1).

In the 17th century, the English physician Thomas Willis described the symptoms of diabetes and observed the presence of sugar in the urine, thus giving the name diabetes mellitus. In the 19th century, researchers began studying the pancreas and its role in blood sugar regulation. Today, T2D is a widespread medical condition worldwide, affecting millions of people. Treatment for T2D usually involves lifestyle changes, such as healthy eating and regular exercise, along with medications that help lower blood glucose levels. Research continues to provide new insights into the causes and treatment of T2D, with the aim of improving patients' quality of life and reducing the complications associated with the disease(2).

T2D treatment nowadays has become more and more accessible, being a common disease, but which has devastating effects on the whole body over a long period of time and ultimately leads to macroangiopathic and microangiopathic complications.

T2D treatment may involve lifestyle changes such as weight loss, healthy eating and regular exercise. Medicines, including insulin, may also be used to control it blood sugar level. It is important for T2D patients to monitor their blood sugar levels and have regular check-ups with their doctor to prevent long-term complications of the disease, such as kidney, eye and cardiovascular problems.

1.2. DIABETES DIABETES: DEFINITION, CLASSIFICATION AND DIAGNOSIS

Definition: diabetes is a chronic medical condition characterized by high blood glucose levels, known as hyperglycemia. Severe hyperglycemia can cause symptoms such as polyuria (frequent urination), polydipsia (excessive thirst), unexplained weight loss, visual disturbances, and susceptibility to infection. In severe cases, hyperglycemia can lead to complications such as ketoacidosis or non-ketoacidotic hyperosmolar syndrome, which can lead to the risk of death.

Diabetes mellitus is a condition characterized by four main types: type 1 diabetes, type 2 diabetes, gestational diabetes and diabetes associated with other conditions(3).

Chronic hyperglycemia can also affect various body tissues and viscera, such as the eyes, kidneys, nerves, heart, and blood vessels, causing long-term damage and dysfunction. T2D may be caused by disorders of insulin secretion or action, or may be associated with risk factors such as a sedentary lifestyle, obesity, and genetic factors. Treatment of diabetes usually involves lifestyle changes, medication and regular monitoring of blood glucose levels(3).

Table 1 Criteria for differential diagnosis between type 1 and type 2 diabetes

Differential diagnostic criteria between type 1 and type 2 diabetes		
Characteristics	Type 1 diabetes	Type 2 diabetes
CAUSE	The presence of Human Leukocyte Antigen (HLA)	Defect in insulin secretion
Prevalence in the case of the diabetic population	5%-10%	90%
Age of onset	Under 30 years	Over 40 years
Family history	Weak association with the onset of the disease	Strong association with the occurrence of the disease
Obesity	rare	Frequent (60-90%)
Ketoacidosis	Frequency	rare
Insulin secretion	Absence	Different doses of insulin
symptom	• polyuria • fatigue • polydipsia • weight loss • ketoacidosis	• moderate polyuria • fatigue • frequently diagnosed during routine examinations

1.3. TYPES OF TREATMENT IN TYPE 2 DIABETES

The complexity of diabetes treatment has increased in recent years with the introduction of new hypoglycemic agents. Metformin remains the first choice of monotherapy in most cases, but there are several options for multiple combination therapy(4). In addition, combinations of three oral hypoglycemic agents are frequently used. Insulin therapy is usually started with a once-daily dose of long-acting insulin. If basal insulin is no longer sufficient, treatment can be intensified by adding short-acting insulin during meals or combining basal insulin with oral glucose-lowering oral hypoglycemic agents or glucagon-like peptide (GLP)-1 analogues(5). Choosing the best hypoglycemic drug requires consideration not only of its effectiveness in reducing blood glucose levels, but also some aspects, through the effectiveness profile of each drug and economic factors should also be taken into account: modern treatment for type 2 diabetes should aim to control blood glucose levels close to normal.

2. GENERAL INFORMATION ON THE USE OF METFORMIN

2.1 METFORMIN, BRIEF HISTORY, USE, INTERACTIONS

Metformin (dimethylbiguanide) has become the most widely used oral hypoglycemic drug worldwide, being first-line and preferred for the management of type 2 diabetes(2. 3). Metformin being very effective in lowering HbA1c and with a weight loss effect, without increasing the risk of hypoglycemia. You appear firstit was in 1918, being a traditional plant-based medicine known as French lilac or goat's rue, under the scientific name - Galega officinalis, which proved to be rich in guanidine(4). Guanidine derivatives, not including

metformin, were synthesized, and some were used to treat diabetes between the 1920s and 1930s, being discontinued due to toxicity and the increased availability of insulin. Rediscovered in the 1940s in the search for an antimalarial drug, metformin proved useful in treating the flu when it sometimes managed to lower blood sugar. This property was traced by the French physician Jean Sterne, who first reported the use of metformin in the treatment of diabetes in 1957.

Metformin can be more accurately described as antihyperglycemic, rather than hypoglycemic, unlike sulfonylureas, metformin does not cause clinical hypoglycemia, even when administered in high doses. This is very often administered in combination with a sulfonylurea in patients who fail to achieve good glycemic control. Combination therapy of metformin with a sulfonylurea has been shown to lower basal blood glucose levels by up to 20% more than single therapy with a sulfonylurea. The key objective in almost all treatment programs is to ameliorate hyperglycemia, which in reality is only a symptom of impaired glucose metabolism in insulin-sensitive tissues, together with the overproduction of glucose by the liver.(27):

- improvement of symptoms
- prevention and relief of acute complications
- prevention and improvement of chronic complications
- avoiding factors that predispose to mortality
- control of any accompanying disorders
- improving the quality of life

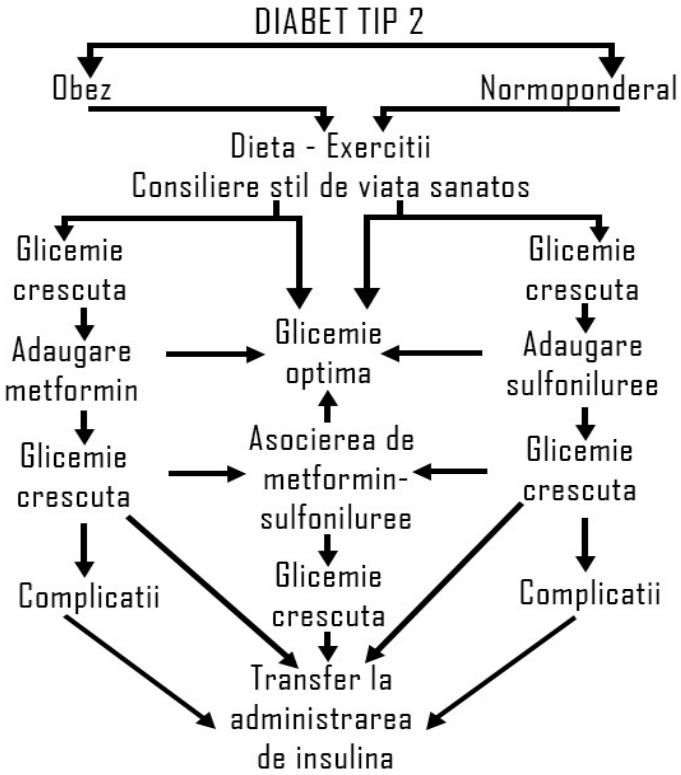

Figure 1. Approaches to the treatment of type 2 diabetes

2.2. THERAPEUTIC USE OF METFORMIN IN PREDIABETES AND DIABETES PREVENTION

Metformin is a drug commonly used to treat type 2 diabetes, but it can also be used to prevent diabetes in people with prediabetes. Prediabetes is a condition where blood sugar levels are higher than normal, but not high enough to be diagnosed as diabetes.

Metformin works by reducing the production of glucose in the liver and by increasing the sensitivity of the body's cells to insulin which is a hormone used to move glucose from the blood into the cells and use it as a source of energy. These actions help control blood glucose levels and prevent diabetes.

Defects in glucose metabolism that underlie type 2 diabetes begin many years before diabetes is diagnosed. Increased insulin secretion initially compensates for the presence of insulin resistance, however, a progressive loss of b-cell mass and b-cell function limits the ability of the pancreas to maintain normal blood glucose by increasing insulin secretion. The early manifestations of prediabetic dysglycemia represent one or both of the following:

- reduced glucose tolerance, in which postprandial glucose control is impaired, but fasting glucose is normal;
- alteration of fasting glucose, is a chronic increase that occurs in the absence of a deterioration of postprandial glucose control.

Table 2 shows the commonly accepted diagnostic criteria for the diagnosis of prediabetes based on glycemic index measurements. A simple blood test is sufficient to diagnose impaired fasting glucose, while a 75 g oral glucose tolerance test (OTG) is required for the diagnosis of impaired glucose tolerance.

2.4. CELLULAR AND MOLECULAR ACTIONS OF METFORMIN

Metformin is water-soluble and is available as metformin hydrochloride, which is its commonly used pharmaceutical form. The chemical formula of metformin hydrochloride is C4H11N5 • HCl.

Metformin is currently the first-line drug for the treatment of type 2 diabetes and is prescribed to at least 120 million people worldwide. It is considered an anti-hyperglycemic agent because it lowers blood glucose concentration in type 2 diabetes without causing severe hypoglycemia. Metformin is also frequently described as an insulin sensitizer, leading to a reduction in insulin resistance and a significant decrease in postprandial plasma insulin levels. The improvement of insulin sensitivity by metformin could be attributed to its positive effects on insulin receptor expression and tyrosine kinase activity. In addition, metformin may exert its beneficial metabolic actions in part by modulating several components of the incretin axis. Evidence from clinical trials and animal models suggests that the main function of metformin is to decrease hepatic glucose production, mainly by inhibiting gluconeogenesis. Several mechanisms have been proposed to explain this inhibitory action, including changes in enzyme activities or a reduction in hepatic uptake of gluconeogenic substrates. The preferential action of metformin in hepatocytes is due to the predominance of expression of the organic cation transporter 1 (OCT1), which has been shown to facilitate the cellular uptake of metformin. Furthermore, metformin accumulation in the liver was found to be higher than in other tissues, reaching high micromolar concentrations in the periportal area mainly by inhibiting gluconeogenesis. Several mechanisms have been proposed to explain this inhibitory action, including changes in enzyme activities or a reduction in hepatic

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Metformin inhibits hepatic gluconeogenesis and improves insulin sensitivity by activating AMPK. It is important to note that metformin has been shown to inhibit hepatic gluconeogenesis in the absence of AMPK. Changes in redox status and gut microbiota, as well as GLP-1 secretion by metformin, have been implicated in AMPK-independent mechanisms for glucose lowering. AMPK: AMP-activated kinase, GLP-1: glucagon-like peptide-type(35). This unique property of metformin causes a decrease in NADH oxidation, proton pumping into the inner mitochondrial membrane, and the rate of oxygen consumption. Ultimately, this leads to a reduction in the proton gradient and a decrease in proton-driven ATP synthesis from ADP and Pi(36).

THE SPECIAL PART

4. EXPERIMENTAL STUDY

4.1. INTRODUCTION – EXPERIMENTAL CONTEXT

Type 2 diabetes mellitus is characterized by hyperglycemia and dyslipidemia as a result of the inability of islet β-cells to secrete insulin. This pathology has become a burden on modern society due to the explosion in the number of cases worldwide and multivisceral lesions(45). Clinical observations have shown that the low life expectancy of diabetic patients is not only related to vascular complications and kidney disease, but also to liver damage. The most common liver complications associated with diabetes are liver cirrhosis and hepatocellular carcinoma(46).

At the same time, the part of liver damage can be associated with the metabolic syndrome, which represents an association of metabolic abnormalities that increase the risk of cardiovascular events. The prevalence of metabolic syndrome in patients with arterial hypertension is very high.

In vitro and in vivo studies have revealed the potential antitumor effects of metformin, and the drug is currently being tested in a number of clinical trials(52).

Colorectal cancer (CRC) has one of the highest incidences among cancers, ranking third globally. With 950,000 deaths estimated in 2020 by the GLOBOCAN study, CRC ranks second in terms of cancer mortality(53).

4.2. RESEARCH OBJECTIVES

The main objectives of this thesis were to determine the complications of diabetes and metformin on the cardiac and digestive systems. We tried to observe the action on the digestive system through in vitro studies, namely the action of metformin in the most discussed areas, namely on the hepatocyte cell line, but also its action on colon cancer cells and a healthy colon cell line, in order to better understand its biological effects, both the cell morphology and its impact on the nuclei were examined. In the case of the effect on the hepatocyte cell line was to evaluate the in vitro and in ovo effect of metformin using five concentrations (0.5, 1, 1.5, 2 and 2.5 mM). Thus, in vitro, the effect exerted by metformin on the hepatocyte cell line - HepaRG, was evaluated in terms of viability, cell morphology, influence on the structures and numbers of nuclei and mitochondria, and on the ability of cell migration. The in ovo biocompatibility and irritant and anti-irritant potential of metformin were assessed using the chicken chorioallantoic membrane (HET-CAM) assay.

We performed experiments at the cellular level to assess how metformin works. Next, we wanted to investigate the impact of diabetes on the development of the cardiovascular system, so we conducted studies on a group of patients. Our objective was to examine whether there is a relationship between subclinical left heart dysfunction, different grades of non-alcoholic fatty liver disease (NAFLD) and the presence of metabolic syndrome in patients diagnosed with type 2 diabetes.

4.3. MATERIALS AND METHODS

4.3.1. THE EFFECT OF METFORMIN ON THE HEPATOCYTE CELL LINE

4.3.1.1. Reagents

Metformin hydrochloride, trypsin-EDTA solution, phosphate buffered saline (PBS), dimethylsulfoxide (DMSO), fetal calf serum (FCS), penicillin/streptomycin, bovine pancreatic insulin, hydrocortisone 21-hemisuccinate sodium, and MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma Aldrich, Merck KgaA (Darmstadt, Germany). Germany). Cell culture media, William's E Medium (Gibco™; 12551032) were purchased from Gibco, Waltham, Massachusetts, USA). All reagents were analytically pure and suitable for use in cell culture.

4.3.1.2. Cell culture

The effect of Metformin on hepatocytes was studied in the HepaRG cell line supplied by Gibco as frozen vials - HepaRG ™ Cells (catalog number: HPRGC10). The cell line was grown in specific culture medium represented by William's E medium containing 4 µg/mL bovine pancreatic insulin, 5 µM Hydrocortisone 21-hemisuccinate sodium, 10% FCS, and 1% penicillin (100 U/mL)-streptomycin (100 µg/mL) mixture. During the experiment, cells were maintained at standard temperature (37°C) and 5% CO2 .

4.3.1.3. Assessment of cell viability

The MTT method was used to determine cell viability. For this purpose, the cells were cultured in 96-well plates in a number of 1x104 cells/well. After reaching approximately 90% confluence, the cells were stimulated with five different concentrations of Metformin (0.5, 1, 1.5, 2 and 2.5 mM) for two time intervals, 24 and 72h. Test concentrations of metformin hydrochloride were prepared in the culture medium. After these time intervals, the stimulation medium was removed and replaced with fresh medium (100 µL/well). 10 µL of MTT reagent was added to each well, and the plates were placed in an incubator at 37 °C for 3 h.

After this time, the solubilization solution was added in a volume of 100 µL/well, and the plates were incubated for 30 minutes at room temperature, protected from light. Subsequently, the measurement of absorbances at two wavelengths of 570 and 630 nM was performed using Cytation 5 (BioTek Instruments Inc., Winooski, VT, USA).

4.3.1.4 Cell morphology

To determine possible Met-induced effects on the morphology and confluence of HepaRG cells, microscopic evaluation was performed by photographing the cells under bright field illumination after the time interval of 24 and 72 h. Images were analyzed using Gen5™ Microplate Data Collection and Analysis Software (BioTek Instruments Inc., Winooski, VT, USA).

4.3.1.5 Immunofluorescence

For immunofluorescence visualization of cellular components, HepaRG were cultured in 12-well plates at 1x105 cells/well, and after reaching an appropriate confluence of approximately 90%, were stimulated for 72 h with Metformin (0.5, 1, 1.5, 2 and 2.5 mM).

After 24 h, cells were washed with ice-cold PBS, then fixed with 4% paraformaldehyde by keeping at room temperature for one hour. Cell permeabilization was performed with 2% Triton X in PBS.

Then, a solution of 30% FBS in 0.01% Triton X was used for one hour to block the action of 2% Triton X. Cells were incubated at 4 °C overnight with mitochondrial marker Anti-COX IV antibody (ab33985) at one dilution. of 1:500 for visualization of mitochondria.

The next day, the primary antibody was washed with 0.01% Triton X solution in PBS and the secondary antibody specific for the mitochondrial marker COX IV - Donkey Anti-Goat IgG H&L (Alexa Fluor® 488 - ab150129) was added and the plate was kept for 2 hours at room temperature and protected from light. After this time, 4',6-diamidino-2-phenylindole (DAPI) staining was added to visualize the nuclei. Images were then captured with Cytation 1 and processed using Gen5™ Micro-plate Data Collection and Analysis Software (BioTek® Instruments Inc., Winooski, VT, USA).

4.3.1.6 Wound healing test

The wound healing (scratch) assay was used to evaluate the effect of metformin on cell migration. For this purpose, cells were cultured in 24-well Corning plates (1x105 cells/well) and an automated scratch was made in the middle of each well using the AutoScratch™ Wound Making Tool provided by BioTek® Instruments Inc., Winooski, VT, USA, according to the manufacturer's recommendations. The cells were then stimulated with the five concentrations of Metformin (0.5, 1, 1.5, 2 and 2.5 mM) for a period of 24 h. Photographs (4x magnification) were taken both at the start of the experiment (0h) and after completion of stimulation (24h) with Cytation 1 and were processed using Gen5™ Microplate Data Collection and Analysis Software (BioTek® Instruments Inc., Winooski, VT, USA).(71).

4.3.1.7 Chorioallantoid membrane (CAM) test

Hen (Gallus gallus domesticus) eggs obtained from a local farmer were used to evaluate the effect of metformin on the vascular plexus. They were disinfected with 70% (V/V) alcohol and, after being dated, placed in the incubator under standard conditions of temperature (37°C) and humidity, in a horizontal position. The eggs were then prepared for the experiment as follows:

on the 4th day of incubation, a perforation was made at the level of the shell through which a volume of 7 ml of albumen was extracted, in order to favor the detachment of the membrane from the internal shell of the egg;

On the 5th day, a window was cut at the upper level of the egg to allow visualization of the chorioallantoic membrane. The cut window was then covered with adhesive tape and the eggs were placed in the incubator until the day the experiment began.

4.3.1.8 Hen egg test - chorioallantoic membrane (HET-CAM)

The Chicken Egg Test (HET-CAM) method was applied to evaluate the biocompatibility and irritant potential at the vascular level. The experiment was performed on the 9th day of incubation.

For this purpose, Metformin was tested at 2 mM, at which the most beneficial effects were previously observed in vitro.

In parallel, a negative control - H2 O and a positive control - 1% sodium dodecyl sulfate (SDS) were used. Both the test sample and the two controls were applied to the chorioallantoic membrane in a volume of 600 µL so that the entire surface of the membrane was covered. Changes observed at the vascular level were: vascular lysis (L), coagulation (C) and hemorrhage (H). These changes were observed for a period of 5 minutes after sample application using a stereomicroscope (Discovery 8 stereomicroscope, Zeiss, Göttingen, Germany), and images were taken (Axio CAM 105 color, Zeiss) before and after 5 minutes of sample application.

To quantify the potential irritant effect, the irritation score (IS) was calculated using the formula(71):

$$IS = 5 \times \frac{301 - H}{300} + 7 \times \frac{301 - L}{300} + 9 \times \frac{301 - C}{300}$$

where H represents hemorrhage, L represents vascular lysis, and C represents intravascular coagulation.

Depending on the value of the irritation score, substances can be classified into three categories, as follows:

Non-irritating (IS = 0 - 0.9);

Irritating (IS = 1 - 8.9)

Severe irritant (IS = 9 - 21) (97).

4.3.1.9 Determination of anti-irritant potential

To determine the potential anti-irritant effect, eggs were prepared as previously described and the method applied was as described above(71)and adapted to our laboratory conditions. Thus, to determine the anti-irritant potential, metformin was tested at a concentration of 2 mM, previously tested in the HET-CAM assay, together with the negative control represented by H2 O. The samples were applied to the chorioallantoic membrane in a volume of 600 µL, and then the eggs were placed in the incubator for a period of 3 h so that the solutions were absorbed by the membrane. After this time, the eggs were treated with 300 µL of 1% sodium dodecyl sulfate (SDS), and the vascular plexus was examined for changes similar to those previously observed by the HET-CAM method (lysis, stasis, and hemorrhage). The changes were observed for a period of 5 minutes, and photographs were taken before and after the application of the samples. The same parameters from the HET-CAM test were followed (H,

$H_{AI} = \frac{H}{H_{SDS}}$; (hemorrhage time after pretreatment with 2 mM Met and addition of 1% SDS / hemorrhage time without pretreatment with 2 mM Met);

$L_{AI} = \frac{L}{L_{SDS}}$; (time of vascular lysis after pretreatment with 2 mM Met and addition of 1% SDS / time of vascular lysis without pretreatment with 2 mM Met);

$C_{AI} = \frac{C}{C_{SDS}}$; (vascular coagulation time after 2 mM Met pretreatment and addition of 1% SDS/vascular coagulation time without 2 mM Met pretreatment).

4.3.2. THE EFFECT OF METFORMIN ON COLON CANCER CELLS AND A HEALTHY COLON CELL LINE

4.3.2.1. Reagents

Analytical pure metformin hydrochloride, trypsin-EDTA solution, phosphate buffered saline (PBS), dimethylsulfoxide (DMSO), fetal calf serum (FCS), penicillin/streptomycin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma Aldrich, Merck KgaA (Darmstadt, Germany). Eagle's Minimum Essential Medium (EMEM, ATCC ® 30- 2003™) and McCoy's 5a Medium Modified (ATCC ® 30- 2007™) were purchased from ATCC (American Type Culture Collection).

4.3.2.2. Cell culture

The normal colon cell line CCD 841 CoN (CRL-1790™) and the cancer cell lines HCT 116 (CCL-247™) and HT-29 (HTB-38™) were purchased from ATCC. CCD 841 CoN cells were cultured in EMEM supplemented with 10% FCS and 1% penicillin (100 U/mL) - streptomycin (100 µg/mL) mixture, while HCT 116 and HT-29 cells were cultured in McCoy's modified 5a medium supplemented with 10% FCS and 1% penicillin (100 U/mL) - streptomycin (100 µg/mL) mixture. All cell lines were maintained under standard conditions in an incubator at 37°C and 5% CO2. â

4.3.2.3. Assessment of cell viability

To determine the cell viability of CCD 841 CoN, HCT 116 and HT-29, the MTT assay was performed 72 h after Met treatment according to the following protocol [2, 3]. Briefly, 96-well plates were seeded with 1 × 104 /200 µL cells/well and, after reaching 90% confluence, metformin test solutions were added. A stock solution of metformin was prepared by dissolving in water for injection, followed by the preparation of five different concentrations in culture media: 5, 10, 25, 50, 75 mM. After 72 h, the test solutions were replaced with 100 µL of fresh culture medium, followed by the addition of 10 µL of MTT reagent. The plates were left in an incubator at 37°C for 3 h. After the incubation time, 100 µL of solubilization solution was added and the plates were left at room temperature, sheltered from light. Absorbance was read at 570 and 630 nm using the Cytation 5 instrument (BioTek Instruments Inc., Winooski, VT, USA). The second wavelength was used for background signals.

Data obtained were expressed as percentage (%) of viable cells normalized to control cells.

4.3.2.4. Cell morphology

Microscopic analysis was performed to identify the impact metformin had on the normal colon cell line CCD 841 CoN and on the colorectal cancer cell lines HCT 116 and HT-29. After the same time interval, cells were examined under bright field illumination using Cytation 1 (BioTek Instruments Inc., Winooski, VT, USA).

Gen5 ™ Microplate Data Collection and Analysis Software (BioTek Instruments Inc., Winooski, VT, United States) was used to process the images.

4.3.2.5 Immunofluorescence staining

The cells were subjected to immunolabeling to obtain new information about the mechanism by which metformin exerts its effect. In this sense, Dapi was used to visualize the nuclei of the cells(72).

The analysis was performed in a manner similar to that previously described(73). CCD 841 CoN, HCT 116 and HT-29 cells were seeded at a density of 1 × 105 cells/well in 12-well plates and, after reaching 90% confluence, were treated with 5 and 75 mM Met for 72 h.

After the desired treatment time, cells were washed with cold PBS and fixed with 4% paraformaldehyde. After being left for 30 min at 4°C, they were washed again with PBS.

Permeabilization was performed with a 2% TritonX solution, the plate was left for 30 min at room temperature, after which 0.01% TritonX was used to wash the cells before the addition of the blocking solution. With the blocking solution, the cells were left at 4°C for 30 min, after which they were washed again with 0.01% TritonX. The final steps consist of adding 300 µL/well of Dapi dye, keeping the plate for 15 min at 4°C. After washing cells with PBS, images were captured with an Olympus IX73 inverted microscope (Olympus, Tokyo, Japan) and analyzed with cellSens Dimensions v.17 software (Olympus, Tokyo, Japan).

4.3.2.6 Statistical analysis

Results are expressed as means ± SD (standard deviation), one-way ANOVA followed by Dunett's multiple comparison post-test. The software used for statistical analysis was Graph-Pad Prism version 9.4.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Statistically significant differences between data are marked with * (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

4.3.3. EXPLORING THE INTERCONNECTION BETWEEN SUBCLINICAL LEFT HEART DYSFUNCTION AND LIVER STIFFNESS IN PATIENTS WITH METABOLIC SYNDROME, DIABETES MELLITUS AND NON-ALCOHOLIC FATTY HEPATIC STEATOSIS

4.3.3.1. Selection of subjects

The departments of cardiology and internal medicine at the Victor Babeș University of Medicine and Pharmacy in Timișoara, secondary care centers, conducted this observational research between January 2021 and August 2022. We consecutively recruited adult patients with MetS and LV ejection fraction (EF) ≥ 50% who agreed to have liver elastography performed as part of their evaluation. They were divided into two groups based on whether or not they had diabetes. In addition to 2D-traditional and STE of the left atrium and left ventricle, all individuals underwent VCTE and CAP. Participants had to be at least 18 years old and have MetS to be considered for inclusion in the study. Exclusion criteria included: known coronary artery disease, peripheral arterial disease,

Before cardiac and hepatic ultrasound investigations, all patients underwent a clinical history review, a complete physical examination, a 12-lead resting electrocardiogram, and laboratory tests.

MetS was defined by the 2006 IDF parameters as central obesity (waist circumference ≥80 cm in women, ≥94 in men), along with any two of the following requirements: systolic blood pressure of at least 130 mmHg or blood pressure diastolic blood pressure of at least 85 mmHg, or treatment for high blood pressure(74), fasting plasma glucose ≥100 mg/dL or treatment for diabetes; triglyceride level ≥ 150 mg/dL or treatment for this lipid disorder; high-density lipoprotein cholesterol level < 40/50 mg/dL (men/women)(74).

Diabetes was diagnosed when fasting plasma glucose exceeded 126 g/mL, twice on two nonconsecutive days, when glycated hemoglobin (HbA1c) was ≥6.5%, or when the patient was receiving oral hypoglycemic medication and/or insulin. In 4 out of 15 patients with prediabetes (fasting plasma glucose 100-126 g/mL and HbA1c 5.7-6.4%) were included in the non-diabetic group(74).

Hypertension was diagnosed when blood pressure was ≥140/90 mmHg and/or the patient was taking medication for hypertension(75).

4.3.3.2. Vibration Controlled Transient Elastography (VCTE) and Determination of Controlled Attenuation Parameters (CAP)

The same investigator performed VCTE after a fast of more than 4 hours using a FibroScan® instrument (EchoSens, Paris, France). 3.5 MHz or 2.5 MHz probe was used according to European standards(76). The PAC cut-offs used to differentiate degrees of steatosis were as follows: - CAP: S1 (mild)-274 dB/m, S2 (moderate)-290 dB/m and S3 (severe)-302 dB/m(76). In each patient, the examiner performed 10 measurements of liver stiffness (LSM), and the median value was calculated. Measurements with a median value and an interval/median ratio with interquartile range < 30% were considered valid(77). LSM was expressed in kilopascals (kPa).

The VCTE thresholds used to separate grades of fibrosis were: VCTE: F2: 8.2 kPa, F3: 9.7 kPa, and F4: 13.6 kPa(78). The same researcher used a VIVID 5S, GE phased array ultrasound oscilloscope with a 3.5 MHz probe for conventional echocardiography.

Heart chamber diameters and volumes were assessed according to the recommendations of the American Society of Echocardiography(79). LV and LA volumes were calculated using the biplane Simpson technique from the apical 4- and 2-chamber images. LV ejection fraction (EF) was determined using the Simpson method. LV diastolic function was assessed using pulsed Doppler ultrasound in apical 4-chamber and 2-chamber images with the sample volume placed at the extremity of the mitral valves.

At the end of the T wave, which precedes the opening of the mitral valves, the maximum volume of the LA (LAVmax) was documented in the 4-chamber and 2-chamber apical events. Minimum LA volume (LAVmin) was assessed in the early stages of ventricular diastole, after the QRS complex, as soon as the mitral valves closed. LA total cerebral volume (tLASV) was calculated as the difference between LAVmax and LAVmin. LA ejection fraction (EF, %) was estimated using the formula 100× [LAVmaxLAVmin]/LAVmax(80).

4.3.3.3. 2D follow-up ultrasound (2D-STE)

The echocardiographic study was performed using Vivid Echo PAC 201 software (GE Medical System) at a rate of 60–90 frames per second. To obtain apical 4- and 2-chamber images, at least three consecutive cardiac cycles were captured during a respiratory pause. Video analysis was performed later, offline. The atrial endocardium and epicardium were traced and adjusted mechanically by the examiner. The software divided the atrium into six distinct regions. The maximum left atrial pool (LA-pool) voltage was measured immediately before mitral valve opening, while the maximum left atrial pumping voltage (LA-pump) was measured just before the P-wave (according to Figure 11).(81,82).

For assessment of ventricular myocardium strain, the Echo PAC 201 software was set at 70–80 frames per second (according to study 74). The program divided the ventricle into six distinct segments (as per Figure 12). 2D-ST images of these six segments were examined in 4-, 3-, and 2-chamber apical incidences. The average of the values obtained from the 18 investigated segments was used to calculate the maximum global longitudinal stress (GLS). Patients with low quality echocardiographic images were excluded from the analysis. The reference parameters for the diagnosis of left atrial dysfunction were as follows: value < 0, 8 for E/A ratio and IVRT duration (time interval between A wave and E wave) > 100 ms for left ventricular (LV) diastolic dysfunction; left ventricular ejection fraction (LVEF) < 50% and peak global longitudinal stress (GLS) < -18% for left ventricular (LV) systolic dysfunction(83).

4.4. RESULT

4.4.1. EXPLORING THE IMPACT OF METFORMIN ON HEPATOCYTE CELL VIABILITY

4.4.1.1 Assessment of cell viability

To investigate the effect of Metformin-induced hepatocytes, we evaluated the viability of HepaRG cells at intervals of 24 and 72 h after stimulation with Metformin at different concentrations: 0.5, 1, 1.5, 2, and 2.5 mM.

Thus, our results indicate that Metformin, at the 24-hour time interval, does not cause a decrease in cell viability; on the contrary, at the concentration of 2 mM, a significant increase in cell viability is observed.

At the 72-hour time point, the same trend is observed regarding the influence on cell viability. Thus, at the first four tested concentrations (0.5, 1, 1.5 and 2 mM) an increase in cell viability is observed depending on the concentration, while at the concentration of 2.5 mM a decrease in cell viability is observed, but not less than the control, the viability being approximately 100% in this case. However, the highest value of cell viability was recorded at a concentration of 2 mM, being about 171%.

The effect of Metformin on the morphology and confluence of HepaRG cells was evaluated and monitored by photographing the cells after two time intervals, 24h and 72h. After a 24-hour stimulation, the cell morphology confirms the results previously obtained in the cell viability assay.

Regarding the 72-hour time frame, the results obtained are similar to those observed in the case of the cell viability test. Thus, at the first four concentrations tested, a dose-dependent increase in confluence and cell number was observed, while at the highest concentration tested, 2.5 mM, there was a slight decrease in cell confluence.

4.4.1.3 Immunofluorescence

To evaluate whether metformin can exert effects on the morphology of the nucleus and mitochondria, the immunofluorescence method was applied. Thus, regarding the core structure, it is not influenced by any of the tested concentrations. Even at the highest concentration tested, no significant changes such as condensation or diminution of number are observed. Similarly, in the case of mitochondria, Metformin treatment does not cause changes in distribution and morphology. Thus, the mitochondria, in the case of the first four concentrations tested, show a uniform distribution, without signs of condensation or decrease in number. Conversely, at the highest concentration tested, a slight condensation of mitochondria around the nuclei is observed

Based on the fact that metformin does not cause vascular irritation and that cell viability is stimulated after metformin treatment, it was decided to evaluate the protective and anti-irritant effects of metformin. Thus, a pretreatment with Metformin and H O2 was performed, followed by irritation with SDS 1%. Pretreatment with H2O does not prevent the occurrence of the vascular irritant effects exerted by SDS, as it is observed that both areas with vascular lysis and areas with hemorrhage are present. On the other hand, pretreatment with 2 mM Met exerts a protective and antiirritant effect, the irritation score obtained in this case (7.29) being significantly lower than that obtained in the case of SDS application without pretreatment. Within five minutes, the presence of small irritant effects at the vascular level was evident, manifested by a slight vascular lysis,

4.4.2. EVALUATION OF THE CYTOTOXIC POTENTIAL OF METFORMIN ON COLORECTAL CANCER CELLS

The current study evaluated the cytotoxic activity of metformin in two colorectal cancer cell lines, HCT 116 and HT-29. A healthy colon cell line was also used to test the effects of Met, namely CCD 841 CoN.

To determine cell viability in all three cell lines used, the MTT assay was performed. After a careful review of the literature, the following concentrations of Met were chosen for testing: 5, 10, 25, 50, 75 mM(54,55,88).

In the healthy colon cell line, 72 h treatment with Met showed no cytotoxic effect. The concentration of 5mM increased cell viability to 101%, while the reported concentrations from 10mM to 75mM decreased cell viability in a concentration-dependent manner, but none of them were less than 79%

The ISO 10993-5:2009 standard states that a compound exerts a cytotoxic effect if it causes a decrease in cell viability by more than 30%. Therefore, metformin does not appear to exert cytotoxicity on normal human colon cells. The colorectal adenocarcinoma cell line, HT -29, showed sensitivity to the treatment. All test solutions decreased cell viability in a manner directly proportional to the concentrations used, but a cytotoxic effect was observed starting at 25 mM, where 69% viability was obtained. 75 mM affected cells the most, with 56% of cells remaining viable after 72 h of stimulation.In the case of HCT 116, the colorectal carcinoma cell line, the therapeutic response was stronger than in the case of HT-29 cells. Again, a loss of a was observedviability as a function of concentration, with metformin causing a cytotoxic effect starting at 10 mM (figure 24). At 75 mM, cell viability decreased to 39%.

For the present study, we chose the MTT test, a colorimetric test that allows the determination of cell viability by determining the mitochondrial function of cells. The dehydrogenase enzyme in viable cells transforms 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide into formazan crystals, which, after solubilization, can be quantitatively measured by spectrophotometry at 570 nm [12]. Results similar to those presented in the present study have been reported in the literature. In a study by Zheng and colleagues, by means of the MTT assay, 20 mM metformin was shown to decrease the cell viability of two colon cancer cells in a time-dependent manner(55). The same results were obtained by Yip et al who tested different concentrations of metformin on colon cancer cells HT -29, SW480 and SW620 for 24, 48 and 72 h. Met showed a time- and concentration-dependent inhibition of cell proliferation.

4.4.3. THE HIGHLIGHTED INTERCONNECTION BETWEEN SUBCLINICAL LEFT HEART DYSFUNCTION AND LIVER STIFFNESS IN PATIENTS WITH METABOLIC SYNDROME, DIABETES MELLITUS AND NON-ALCOHOLIC FATTY LIVER DISEASE

Analyzing the obtained data, we did not identify significant differences between the two groups in terms of age distribution, gender, hypertension prevalence, waist circumference, body mass index, smoking status, the number of components of the metabolic syndrome and drug treatment for lipid disorders and hypertension.

There were no appreciable differences in traditional measures of LV function and structure between the two groups, except for LV diastolic dysfunction, which was more common in subjects with diabetes. We also found no changes in LA diameters, volumes, or ejection fractions between diabetic and non-diabetic MetS patients.

2D-STE, on the other hand, detected subtle LV systolic dysfunction and subtle LA dysfunction in diabetic patients, evidenced by significantly lower overall LV longitudinal strains, lower LA reservoir and pump strains, and increased LA stiffness.

LV diastolic dysfunction, identified by traditional echocardiography, was found in 38 diabetic patients (53%) and in 40 (51%) non-diabetic patients, $p = 0.04$. Subtle LV systolic dysfunction, defined by GLS < 18%, was found in 47 (65%) diabetic patients and 34 (44% non-diabetic subjects $p < 0.0001$). Subtle LA dysfunction, identified by LA stiffness > 0.38 was found in 29 (40%) diabetic MetS patients and in 15 (19%) non-diabetic patients, $p = 0.03$. Diabetic MetS patients had a 1.5-fold increased risk of LV systolic dysfunction (95% CI 1.10 to 2.02, $p < 0.01$) and a 2-fold increased risk of LA dysfunction (95% CI 1.22 to 3.57, $p < 0.01$)

In both univariate and multivariate logistic regression analysis, liver fibrosis ≥ 2 was an independent predictor of subtle LV dysfunction and subtle LA dysfunction in MetS diabetic patients with NAFLD.

We found significant associations between liver fibrosis ≥ 2 with both subtle LV systolic dysfunction and subtle LA dysfunction in diabetic MetS patients. Subclinical LV

systolic dysfunction, assessed by reduced global longitudinal strain (%), was significantly related to liver stiffness (kPa), $p < 0.001$ (Figure31). LA dysfunction, assessed by increased LA stiffness (%), was also significantly associated with liver stiffness (kPa).

4.5. DISCUSSIONS

4.5.1. EVALUATION OF THE ACTION OF METFORMIN ON THE HEPATOCYTE CELL LINE: IMPLICATIONS IN THE PATHOGENESIS OF METABOLIC SYNDROME AND NAFLD.

The results of the present study showed that, in vitro, metformin stimulates cell proliferation and migration, key factors in the repair process.

For a more general picture of the induced effect, the present study evaluated its action in blood vessels, both in terms of biocompatibility and anti-irritant potential, using hen's egg chorioallantoic membrane.

The obtained results showed that Met does not induce irritating effects at the level of the vascular plexus, having an irritation score of 0.7, which indicates that it is a substance without toxic effects. Furthermore, regarding the potential protective role, the pretreatment significantly diminished the irritant effects induced by SDS at the capillary level.

Thus, if in the absence of pretreatment, the irritation score induced by SDS was 19.68, in the case of pretreatment, it decreased to 7.29. These results support previous findings that it increases cell viability and stimulates cell migration with a potential impact on wound healing.

To the best of our knowledge, metformin has not been previously tested for irritant and anti-irritant effect by the HET-CAM method. However, it was previously applied to the chorioallantoic membrane to evaluate the potential effect on angiogenesis. Thus, Wang and his colleagues used the CAM method to highlight its antiangiogenic effect in combination with Pemetrexed(115).

4.5.2. SIGNIFICANT CORRELATION BETWEEN SUBCLINICAL LEFT HEART DYSFUNCTION AND LIVER STIFFNESS IN METABOLIC SYNDROME, DIABETES MELLITUS AND NON-ALCOHOLIC FATTY LIVER DISEASE

Participants in our study were thoroughly examined using hepatic VCTE and CAP to identify and quantify NALD, along with conventional ultrasonography and speckle-tracking to assess cardiac structure and function.

Hepatic steatosis grade S2 and S3 was significantly more frequent than in non-diabetic MetS patients, as shown in Table2, as well as liver fibrosis F2 and F3.

Although LA and LV diameters, volumes, and ejection percentages were not substantially different between MetS participants with and without DM, 2D-STE revealed LA and LV strain characteristics that were significantly worse in the presence of DM, indicating subclinical dysfunction ($p < 0.04$).

According to previous research, the pattern of myocardial deformation is highly related to the severity of myocardial fibrosis determined by cardiac MRI or histopathological samples(122).

Despite evidence that patients with NAFLD are at risk of structural cardiac abnormalities, no link between myocardial and liver fibrosis has been demonstrated.

This is the first study to show this link using left heart deformation patterns in diabetics with MetS.

CONCLUSIONS AND PERSONAL CONTRIBUTIONS

- The present thesis aimed to study possible cardiovascular and digestive implications of metformin and type 2 diabetes
- Currently, we still do not have a correct approach and a total follow-up of complications
- Our findings highlight the possible antitumor effect that metformin exerts in one of the most prevalent types of cancer
- Metformin does not induce toxic effects in hepatocytes
- Metformin causes a stimulation of liver cell proliferation
- Metformin does not induce changes in cell morphology and the structure, organization and number of mitochondria and nuclei
- Metformin stimulates cell migration, with a possible beneficial effect in the healing of toxic and irritant wounds and that it has anti-inflammatory and protective effects against vascular irritation.
- Further studies are needed to determine the possible biological mechanisms underlying this therapeutic action and to fully elucidate the action of metformin on the systems
- Our results underline the importance of liver assessment by methods such as CAP and VCTE, as well as assessment of cardiac function by means of the 2D-STE technique in diabetic patients with metabolic syndrome
- We recommend that evaluation of liver and cardiac function be performed consistently in diabetic patients with metabolic syndrome, as these evaluations can contribute to the early identification of abnormalities and the implementation of an appropriate treatment plan
- Future studies should continue to investigate this complex interconnection between liver, heart and metabolic conditions to improve the management and prevention of cardiovascular complications