„CAROL DAVILA" UNIVERSITY OF MEDICINE
AND PHARMACY, BUCHAREST
DOCTORAL SCHOOL
PHARMACY FIELD

„Evaluation of the antihyperalgesic potential of fixed combinations of active substances"

Ph.D. Thesis Summary

Ph.D. supervisor
PROF. UNIV. DR. SIMONA NEGREȘ

Ph.D. student
CIPRIAN PUȘCAȘU

BUCHAREST
2023

Table of contents

Abbreviations and symbols

AAN	American Academy of Neurology
Acetyl~CoA	Acetyl~Coenzyme A
AGE	Advanced glycation products
AMPK	5' adenosine monophosphate-activated protein kinase
Ang 1	Angiopoietin 1
ATP	Adenosine triphosphate
cAMPc	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
COX-2	Cyclooxygenase-2
DN4	Douleur Neuropathique 4 questionnaire
DNA	Deoxyribonucleic Acid
DPN	Diabetic peripheral neuropaty
ED_{50}	Median effective dose
EFNS	European Federation of the Neurological Societies
G	Gabapentin
GABA	Gamma-Aminobutyric Acid
GBN	Gabapentin
Glucosamine-6-P	Glucosamine-6-phosphat
GSH	Gluthatione
Hb	Hemoglobin
HbA1c	Glycosylated hemoglobin
Hct	Hematocrit
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
i.p.	intraperitoneally
IASP	International Association for the Study of Pain
IGF	Insulin-like growth factor

IHS	International Headache Society
IL-1ß	Interleukine 1ß
IL-6	Interleukine 6
iNOS	Inducible nitric oxide synthase
LANSS	Leeds Assessment of Neuropathic Symptoms and Signs scale
LDL	Low density lipoprotein
M	Metformin
MD	Diabetic control group
MPV	Medium platelet volume
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of rapamycin complex 1
NEFA	Free fatty acids
NF-kB	Nuclear factor kappa B
NGF	Nerve growth factor
NMDA	N-Methyl-D-aspartic acid
NO	Nitric oxide
NOS	Nitric oxide synthases
NPIC	Chemotherapy-induced peripheral neuropathy
OD	Optical density
ORL 1	Oxidized low-density lipoprotein receptor 1
PDE 5	Phosphodiesterase 5
PG	Prostaglandins
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PGI2	Prostaglandin I2
PPARγ	Peroxisome proliferator- activated receptor gamma
RAGE	Receptor for advanced glycation end products
RBC	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive species of oxygen
S	Sildenafil

S.E.M.	Standard error of the mean
S-LANSS	Leeds Assessment of Neuropathic Symptoms and Signs patient self-report of the scale
SRNIs	Serotonin and norepinephrine reuptake inhibitors
TCA	Tricyclic antidepressants
TLR4	Toll-like 4 receptor
TNF-α	Tumor necrosis factor α
TRPV1	Transient receptor potential vanilloid 1
TTX	Tetrodotoxin
UDP-GlcNAc	Uridine diphosphate N-acetylglucosamine
VEGF	Vascular endothelial growth factor
WBC	White blood cells
WHO	World Health Organization

Introduction

According to the WHO, diabetes is "a chronic metabolic disease characterized by elevated blood glucose levels, which over time leads to serious damage to the nerves, kidneys, heart, blood vessels and eyes". Neuropathy is one of the long-term complications of both type 1 and type 2 diabetes, characterized by pain, sensory loss, affecting quality of life and even leading to limb amputation. Worldwide, the International Diabetes Federation estimates that 537 million people have diabetes, and of these, at least 50% develop diabetic neuropathy over time.

Few therapeutic strategies are currently available, with limited success in alleviating pain. According to the Guidelines of the European Federation of Neurological Societies (EFNS) and the American Academy of Neurology (AAN), diabetic neuropathy therapy includes tricyclic antidepressants (amitriptyline), gabapentinoids (gabapentin and pregabalin) and non-selective adrenaline and noradrenaline reuptake inhibitors (duloxetine and venlafaxine) as first-line therapy. Tramadol can be used as second-line therapy, and if topical treatment is preferred, capsaicin is preferred. Strong opioids (morphine and oxycodone) are recommended as third line therapy because of their potential of abuse.

The wide variety of pathophysiological mechanisms involved in the development and progression of diabetic neuropathy makes it difficult to design effective therapeutic strategies. Therefore, in the need to discover new active substances with improved efficacy and fewer side effects, we set out to investigate the effects of two drugs already used for the treatment of hyperglycemia or its long term complications in this PhD thesis. Thus, the research hypothesis started from the following considerations:

- sildenafil, a PDE-5 inhibitor used for the treatment of erectile dysfunction in diabetes mellitus, may have a beneficial effect in diabetic neuropathy, as it improves neurovascular function by significantly increasing the number of functional blood vessels and regional blood flow in the sciatic nerve (this hypothesis is also supported by data from the literature that indicated that diabetic patients with erectile dysfunction treated with sildenafil showed reduced symptoms of neuropathy);
- metformin is an antihyperglycemic agent recommended as first line therapy by current guidelines (American Diabetes Association (2021) and European Association for the

Study of Diabetes (2021)) that relieves pain by activating adenosine monophosphate-activated protein kinase (AMPK), considered a target in the treatment of pain.

The general objectives of this research were the following:

- Validation of the method of inducing diabetic neuropathy by administering alloxan to mice, simultaneously with the monitoring of blood glucose values and blood parameters;
- Evaluation of antihyperalgesic potential in various experimental pharmacological tests for selected compounds in an animal model of diabetic neuropathy. In parallel, we determined fructosamine and hematological parameters, along with the biochemical markers from brain and liver homogenates;
- The 50% mean effective doses (DE_{50}) for the treatment of diabetic neuropathy in mice were determined for sildenafil and metformin;
- Evaluation of the synergism between sildenafil and metformin in an animal model of diabetic neuropathy in mice and rats.

I. The general part

1. General physiopathological aspects of neuropathic pain

The International Association for the Study of Pain (IASP) defines neuropathic pain as "pain caused by an injury or disease of the nervous system" [1]. Neuropathic pain is characterized as constant or intermittent, spontaneous or provoked, and is described as burning, stinging, tingling, itching, stabbing. It can be accompanied by allodynia (appearance of the sensation of pain to a non-painful stimulus) or hyperalgesia (exaggeration of the sensation of pain) [2]. Neuropathic pain is classified as central or peripheral depending on the location.

Diagnosing neuropathic pain can be a real challenge, given its association with other conditions and the absence of a standard diagnostic method. So, not surprisingly, it can be difficult to treat effectively, being associated with significant impairments in quality of life and a substantial economic burden [2].

According to the recent studies, the prevalence of neuropathic pain is as following:

a) Europe-7.7%;

b) Australia-7%;

c) United States of America-5% [2].

Usually, negative and positive sensory symptoms coexist in neuropathic pain. Negative symptoms include attenuation of various somatosensory qualities, such as tactile hypoesthesia or anesthesia, thermal hypoesthesia, hypoalgesia, and loss of vibratory sensation. These symptoms cause discomfort but are not painful. In addition to negative symptoms, spontaneous positive symptoms such as paresthesia and dysesthesia, paroxysmal pain, and continuous superficial pain may occur. Other positive symptoms include hyperalgesia and allodynia, being elicited by various stimuli [3].

Diabetes and its complications are growing challenges for health systems worldwide. According to the International Diabetes Federation, 425 million people worldwide aged ≥ 20 years had diabetes in 2017, and this number is expected to increase to 629 million by 2045 [3].

Diabetic neuropathy is a common and, until recently, largely neglected problem, affecting approximately 50% of patients with diabetes at some point [4-7]. Diabetic neuropathy can be defined as "the presence of symptoms and/or signs due to peripheral nerve dysfunction in people with diabetes after exclusion of other causes" [7]. A major problem with DN is that once it has developed and complications have occurred, it is difficult to treat and patients face an increased risk of amputations associated with increased mortality [8-11].

HbA1c levels and duration of diabetes, along with other metabolic factors such as hypertension and insulin resistance, are predictors of diabetic neuropathy. Likewise, numerous studies have highlighted the presence of obesity in patients with neuropathy, but also hypertriglyceridemia and low levels of high-density lipoproteins (HDL), especially in patients with type 2 diabetes. In addition to these factors, smoking, alcohol and advanced age contribute to the progression of diabetic neuropathy [12-14].

For a better view of the onset and progression of diabetic neuropathy, it is essential to describe the process by which energy is produced by the peripheral nervous system through the use of substrates. In diabetes, the long-chain fatty acid transport system in Schwann cells becomes saturated and acetyl-CoA (acetyl coenzyme A) molecules are converted to acylcarnitine, which is toxic to both Schwann cells and dorsal root ganglion neurons, contributing to the development of diabetic neuropathy along with nerve damage [15]. Elevated glucose levels lead to the metabolism of glucose through one or more metabolic pathways, such as the polyol pathway and the hexosamine pathway, leading to increased ROS and

inflammation, respectively, largely due to mitochondrial dysfunctions [16], which contribute to ongoing damage to the nervous system.

Although many studies suggest that there are no changes in blood flow associated with diabetic neuropathy, poor blood circulation occurs in peripheral nerves that may contribute to the progression of diabetic neuropathy. Poor circulation at the microvascular level leads to additional damage to peripheral nerves, associated with their dysfunction. Diabetes causes ischemia of nerve fibers, which causes an increase in the density of endoneurial capillaries [17].

2. Therapeutic management of neuropathic pain

The management of neuropathic pain focuses on treating the symptoms, and only in certain pathological conditions the etiological causes can be treated, thus relieving the pain [18].

Pharmacological treatment of neuropathic pain includes:

I. First line agents:

1. Tricyclic antidepressants, especially amitriptyline (acts by inhibiting noradrenaline and serotonin, but also has effects on ion channels, adrenergic, histaminergic and cholinergic receptors; side effects include drowsiness, dizziness, dry mouth, orthostatic hypotension and prolongation of the QT interval; they are contraindicated in patients with glaucoma, prostate hypertrophy and cardiac disorders) [18-20].

2. Non-selective serotonin and noradrenaline reuptake inhibitors, especially duloxetine and venlafaxine (act by non-selective inhibition of serotonin and noradrenaline reuptake; side effects include nausea, abdominal pain and constipation, high blood pressure (at high doses for venlafaxine); use with caution in patients with cardiac disorders and liver conditions (in the case of duloxetine) [18-20].

3. Gabapentinoids: pregabalin, gabapentin (their analgesic effect is mainly due to a decrease in central sensitization by binding to the α2-δ subunit of voltage-dependent Ca^{2+} channels; side effects include sedation, dizziness, peripheral edema, weight gain, blurred vision) [18-20].

II. Second line agents:

1. Topical treatment: lidocaine (acts by decreasing spontaneous ectopic discharge by blocking sodium channels; side effects are local, especially skin irritations) and capsaicin (acts

by activating the TRPV1 receptor, leading to desensitization of TRPV1 at the level of epidermal nerve fibers; side effects occur locally - redness, itching) [18-20].

2. Opioids: tramadol and tapentadol (inhibits nociceptive transmission via pre- and postsynaptic μ opioid receptors; tramadol presents a mixed mechanism of action: μ opioid agonist and inhibition of serotonin and noradrenaline reuptake; recommended as second-line agents due to the increased risk of abuse although lower compared to strong opioids) and risk of confusion and drowsiness especially in the elderly; it is used with caution in combination with antidepressants) [18-20].

III. Third line agents

1. Strong opioids: oxycodone and morphine (act as μ opioid receptor agonists; present increased risk of pharmacodependence) [18-20].

2. Botulinum toxin type A (a potent neurotoxin commonly used for the treatment of focal muscle hyperactivity, an effect due to inhibition of synaptic exocytosis and neuronal transmission; the treatment is generally considered safe, although there is limited evidence from long-term studies) [18-20].

II. Personal contributions

3. Working hypothesis and general objectives

Neuropathy is one of the common complications of diabetes that occurs due to damage to the peripheral nervous system. The neuropathy that occurs in diabetes is called diabetic neuropathy and affects about 50% of patients with diabetes.

Erectile dysfunction (ED) has also neurological [21] and microvascular etiology [22] and is closely related to cardiovascular pathologies and its factors of risk, especially diabetes [23-24].

There is no single treatment to prevent or reverse neuropathic changes or to provide total pain relief. Therefore, we believe there is a great need for studies evaluating the most effective drugs or combinations for the management of DNP to maximize pain relief and improve quality of life. Considering these considerations, we proposed to investigate the effects of sildenafil and metformin, administered as monotherapy or in combination for the treatment of peripheral neuropathic pain in diabetic rodents starting from the following observations:

- ✓ The level of PDE-5 increases due to hyperglycemia, and the administration of an PDE-5 inhibitor, such as sildenafil, can improve blood circulation in the sciatic nerve by increasing the expression of cGMP;
- ✓ Metformin, in addition to its antihyperglycemic action by which it improves glycemic status, can also prevent the onset of neuropathy through two mechanisms: activating AMPK (considered a target for pain treatment) and inhibiting mTORC1.

In this PhD thesis, we aimed to evaluate the antihyperalgesic potential of different doses of sildenafil and metformin, but also of the sildenafil-metformin combination in various experimental pharmacological tests and the influence of these drugs on blood tests (hemoleukogram, fructosamine) and different biochemical markers (cytokines pro-inflammatory, nitrites, thiols) from brain and liver homogenates.

4. Investigation of antihyperalgesic potential for different doses of sildenafil and metformin in alloxan-induced diabetic neuropathy in mouse

In the need to discover new active substances with improved efficacy and fewer side effects, recent research showed that treatment of diabetic peripheral neuropathy with sildenafil, a PDE 5 inhibitor, improves sciatic nerve circulation and neurological function in experimental animal models [25-26]. Also, the antihyperalgesic effect of metformin has been studied in animal models in several researches and it has been shown that, in addition to its antihyperglycemic effects, it alleviates pain by inhibiting mTORC and activating AMPK [27-29]. Therefore, sildenafil and metformin work through different mechanisms of action than the drugs that are recommended in diabetic neuropathy according to current guidelines. Furthermore, the repurposing of these two drugs for the management of this pathology could provide therapeutic options with superior efficacy and fewer and less severe side effects.

Materials and methods

The experimental procedures were carried out in accordance with the bioethical rules proposed by Law 43/2014 on the protection of animals used for scientific purposes and Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the

protection of animals used for scientific purposes. The experimental protocol was approved by the Bioethics Commission of the Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania.

A number of 110 diabetic animals were selected, divided into 11 experimental groups (n=10) that received daily treatment for 14 days by oral gavage, as follows: distilled water for the non-diabetic control group (ND), distilled water for the diabetic control group (D), gabapentin 50 $mg \cdot kg^{-1}$ (G50 group), gabapentin 100 $mg \cdot kg^{-1}$ (G100 group), gabapentin 150 $mg \cdot kg^{-1}$ (G1 50 group), metformin 150 $mg \cdot kg^{-1}$ (M150 group), metformin 250 $mg \cdot kg^{-1}$ (M250 group), metformin 500 $mg \cdot kg^{-1}$ (M500 group), sildenafil 1.5 $mg \cdot kg^{-1}$ (S1.5 group), sildenafil 2.5 $mg \cdot kg^{-1}$ (S2.5 group), sildenafil 3 $mg \cdot kg^{-1}$ (S3 group).

The antihyperlagic potential of different doses of sildenafil and metformin was evaluated in the hot-plate test, but also in the cold-stimulus test (tail withdrawal from cooled water) after the induction of diabetes with alloxan. At the end of the experiment, blood samples were collected for evaluation of fructosamine and blood count. The ED_{50} for sildenafil and metformin in the heat and cold hypersensitivity tests were also calculated.

Statistical analysis of the experimental data and their graphical illustration were performed using the GraphPad Prism v.5.00 software package (GraphPad Software, San Diego, CA, USA). The type of data distribution was determined using the D'Agostino-Pearson test. Experimental results were analyzed using the following statistical tests: one-way analysis of variance (ANOVA) test followed by Dunnet's post hoc test for parametric data and Kruskal-Wallis test followed by Dunn's post hoc test for nonparametric data.

Results and discussion

In this study, we observed a significant increase in pain sensitivity in the diabetic control group in both Hot-plate and tail-withdrawal tests when compared to the non-diabetic control. The results obtained are consistent with specialized literature indicating that hyperglycemia-induced oxidative stress is an important mechanism leading to both the development and progression of hyperalgesia and allodynia in rodents [30-31].

In the Hot-plate test, sildenafil produced an antihyperalgesic effect in all diabetic animals treated with different doses. This effect was statistically significant for the doses of 2.5 $mg \cdot kg^{-1}$ and 3 $mg \cdot kg^{-1}$, starting from the 7th day of treatment (Figure 4.1.A). The same antihyperalgesic

effects were obtained for sildenafil in the tail withdrawal test for the same doses, with significant increases in pain reaction latency for doses of 2.5 mg·kg^{-1} and 3 mg·kg^{-1} on day 14 of the experiment (Figure 4.1.B).

Present research showed that in the diabetic groups treated with metformin, pain sensitivity to a cold stimulus (water cooled to 10°C) decreased during evaluation on days 7 and 14 of the experiment. Metformin in doses of 150 mg·kg^{-1} and 500 mg·kg^{-1} showed a significant increase in pain reaction latency when compared to the diabetic control group after 14 days of treatment (Figure 4.1.B). In the Hot-plate test, the antihyperalgesic effect of metformin was observed for all doses. Metformin 150 mg·kg^{-1} demonstrated a significant decrease in pain sensitivity when compared to the diabetic control group after 7 days of treatment, while all doses of metformin showed a significant increase in pain reaction latency compared to the diabetic control group after 14 days of treatment (Figure 4.1.A).

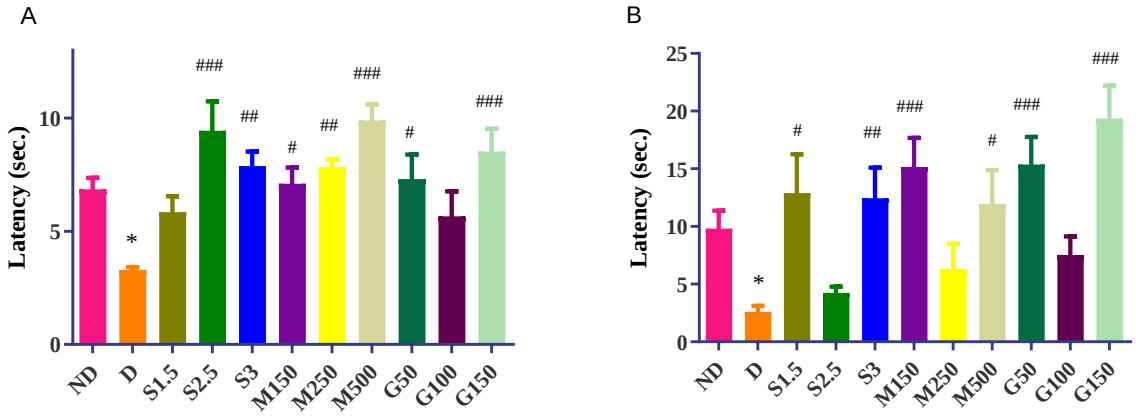

Figure 4.1. (A) Pain reaction latency in the Hot-plate test after 14 days **(B)** Pain reaction latency in the tail withdrawal test after 14 days. Values are expressed as mean ± S.E.M.

Blood tests revealed an increase in WBC values in all diabetic groups when compared to the non-diabetic control group. Significantly lower values were observed in groups treated with metformin 250 mg·kg^{-1} and 500 mg·kg^{-1} when compared to the diabetic control group. We also noted a decrease in RBC, Hb and Hct values in all diabetic groups when compared to the non-diabetic control, although among metformin 250 mg·kg^{-1} and 500 mg·kg^{-1} groups the decrease was not statistically significant. In correlation with other preclinical and clinical studies [32-33], significantly increased MPV values were reported in our study for all diabetic groups

when compared to the non-diabetic mice. In addition, the group treated with metformin at a dose of 250 $mg \cdot kg^{-1}$ showed significantly lower MPV values.

After evaluating the efficacy of the administered drugs in diabetes-induced heat hypersensitivity, we obtained ED_{50} values of 2.07 $mg \cdot kg^{-1}$ for sildenafil, 348.92 $mg \cdot kg^{-1}$ for metformin, and 70.39 $mg \cdot kg^{-1}$ for gabapentin. The calculated ED_{50} values for antihyperalgesic effects in cold hypersensitivity were 2.06 $mg \cdot kg^{-1}$ for sildenafil, 205.90 $mg \cdot kg^{-1}$ for metformin and 104.31 $mg \cdot kg^{-1}$ for gabapentin.

5. Influence of sildenafil-metformin combination on hyperalgesia and biochemical markers in diabetic neuropathy in mice

Experimental research on animal models has demonstrated the antihyperalgesic effect of sildenafil, which improves neurovascular function by significantly increasing the number of functional blood vessels and regional blood flow in the sciatic nerve [26]. On the other hand, metformin, an antihyperglycemic agent, prevented the onset of peripheral neuropathy by activating AMPK [34]. Byrne et al. demonstrated that metformin reduced hyperalgesia in fructose-treated rats, suggesting that its antihyperalgesic effect is not entirely dependent on its hypoglycemic action [35]. In addition, Deftu et al. pointed out that metformin activates AMPK, which consequently modulates the activity of the E3 ubiquitin ligase NEDD4-2 and the expression of voltage-gated sodium channels (NaVs) [36].

It is well known that TNF-α is involved in the pathophysiology of chronic pain [37]. Also, in the case of diabetic patients, inflammation occurs in nerve tissues and several studies have shown an increase of pro-inflammatory cytokines in their blood [34-35]. Besides TNF-α, IL-6 production is also increased in diabetic patients with neuropathy [38].

iNOS is a proinflammatory enzyme that generates intracellular free radicals and increases NO production [39]. Consequently, NO contributes to the progression of neuropathic pain by directly affecting injured peripheral axons and acting as a signaling molecule in the medullary dorsal horn [40].

Materials and methods

The experimental procedures were carried out in accordance with the bioethical rules proposed by Law 43/2014 on the protection of animals used for scientific purposes and Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the protection of animals used for scientific purposes. The experimental protocol was approved by the Bioethics Commission of the Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania.

In this study, we used 100 diabetic mice and 20 non-diabetic mice divided into 6 equal groups (n=20) that received treatments daily for 14 days by oral gavage as follows: non-diabetic control group (ND) – distilled water 0.1 $mL \cdot kg^{-1}$; diabetic control group (D) - distilled water 0.1 $mL \cdot kg^{-1}$; GBN group - gabapentin 100 $mg \cdot kg^{-1}$; S1.5+M150 group - sildenafil 1.5 $mg \cdot kg^{-1}$ + metformin 150 $mg \cdot kg^{-1}$; S2.5+M250 group– sildenafil 2.5 $mg \cdot kg^{-1}$ + metformin 250 $mg \cdot kg^{-1}$; S3+M500 group- sildenafil 3 $mg \cdot kg^{-1}$ + metformin 500 $mg \cdot kg^{-1}$.

We evaluated initial, at 7 and 14 days blood glucose, heat and cold hypersensitivity, and at the end of the experiment we determined TNF-α, IL-6 and the concentration of nitrites on brain and liver homogenates.

Statistical analysis of the experimental data and their graphical illustration were performed using the GraphPad Prism v.5.00 software package (GraphPad Software, San Diego, CA, USA). The type of data distribution was determined using the D'Agostino-Pearson test. Experimental results were analyzed using the following statistical tests: one-way analysis of variance (ANOVA) test followed by Dunnet's post hoc test for parametric data and Kruskal-Wallis test followed by Dunn's post hoc test for nonparametric data.

Results and discussion

One of the most common complications of diabetes is erectile dysfunction. The meta-analysis by Kouidrat et al. showed that the incidence of erectile dysfunction in diabetic patients was 52.5% and its prevalence was about 3.5 times higher than in non-diabetic patients [41]. The efficacy, safety, and improvement in quality of life in patients with type 2 diabetes and erectile dysfunction taking sildenafil are well established [42-43].

The importance of metformin in the treatment of type 2 diabetes is well established by the American Diabetes Association and the European Association for the Study of Diabetes

[44], being recommended as first-line pharmacological therapy. Given that these two drugs, sildenafil and metformin, are commonly prescribed to maintain glycemic control and correct erectile dysfunction in patients with type 2 diabetes, we set out to investigate whether the combination of the two substances can correct diabetic neuropathy, a common complication of this disease.

In the Hot-plate test, all 3 sildenafil-metformin combinations showed a significant decrease in pain sensitivity when compared to the diabetic control group, both after 7 and 14 days of treatment. On the 7th day of the experiment, the S3+M500 combination demonstrated the most intense antihyperalgesic effect, while after 14 days of treatment, the S2.5+M250 group showed a greater increase in the pain reaction latency(Figure 5.1.A). In the cold stimulus test, the antihyperalgesic effect of all sildenafil-metformin combinations observed in the Hot-plate test is supported, with the S3+M500 group registering the highest pain reaction latency when compared to the diabetic control after 14 days of treatment (Figure 5.2.A).

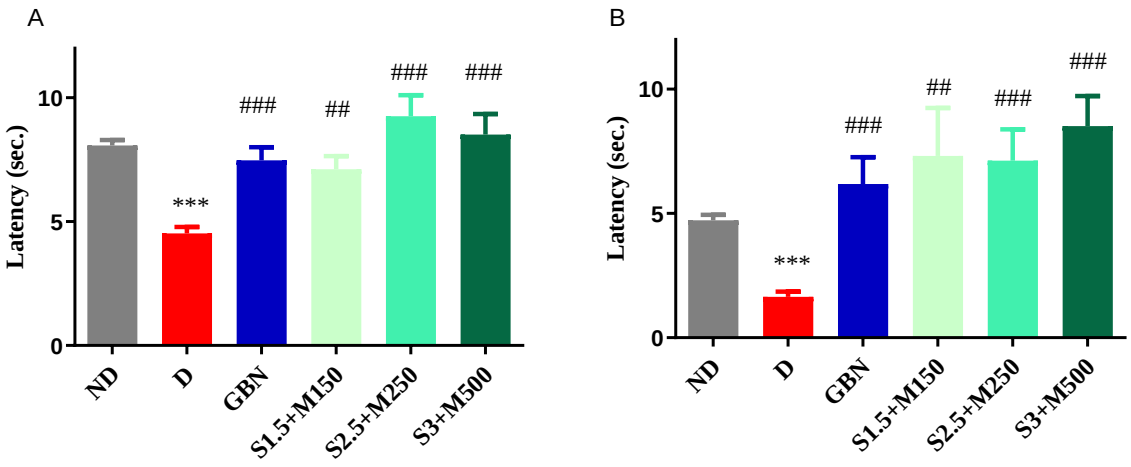

Figure 5.1. (A) Pain reaction latency in the Hot-plate test after 14 days **(B)** Pain reaction latency in the tail-retraction test after 14 days. Values are expressed as mean ± S.E.M

Regarding the level of IL-6, the groups treated with gabapentin and the S2.5+M250 combination recorded significant decreases in the brain tissues when compared to the diabetic control group. For comparison, in liver tissues, the diabetic groups treated with gabapentin and the S1.5+M150 and S2.5+M250 combinations showed non-significant decreases in the level of

IL-6 when compared to the diabetic control group after 14 days of treatment. After 14 days of treatment, nitrites concentration was significantly decreased for the S2.5+M250 and S3+M500 combinations in brain tissues when compared to the diabetic control group, while all diabetic groups receiving treatment with gabapentin and all sildenafil-metformin combinations demonstrated significant decreases of nitrites levels in liver when compared to the diabetic control, suggesting a lower NOS activity.

6. Evaluation of the synergism of the combination between sildenafil and metformin in an animal model of alloxan-induced diabetic neuropathy

In recent years, the therapeutic approach of a pathology with a single drug has slowly shifted towards combination therapy that uses a combination of several active substances. This change occurred following observations that highlighted the limited efficacy of monotherapy in chronic diseases, the emergence of treatment resistance, but also numerous severe adverse effects [45]. The latest results demonstrated that combination therapy could effectively cure chronic diseases with complex etiology and pathophysiology, such as diabetes, cancer, AIDS [46-49].

The purpose of this study was to evaluate the synergism between metformin and sildenafil in an animal model of alloxan-induced diabetic neuropathy based on the results obtained following the individual administration of the 2 substances (Chapter 4) and the association between the 2 drug (Chapter 5) in the heat and cold hypersensitivity tests.

Materials and methods

The experimental analysis was followed by the evaluation of synergism between sildenafil and metformin on neuropathic pain. The synergism was evaluated cumulatively for the 3 combinations between the 2 drugs, for both types of tests based on thermal stimuli for 7 and 14 days respectively after the administration of the substances, the algorithm being thus implemented a total of 4 times. Synergism evaluation was implemented in the Python programming language, version 3.9.2, through the synergy library [50].

Results and discussion

Table 6.1. illustrates the results obtained regarding the evaluation of the synergism between sildenafil and metformin by means of the MuSyC algorithm.

Table 6.1. The results obtained for the evaluation of synergism between sildenafil and metformin

Indicator	**Case 1** **Interpretation**	**Case 2** **Interpretation**	**Case 3** **Interpretation**	**Case 4** **Interpretation**
Beta (β)	0.07 Synergism	4.44 Synergism	-0.01 Antagonism	-0.25 Antagonism
Alpha-12 (α_{12})	0.57 Antagonism	Undefined	1.16 Synergism	0.33 Antagonism
Alpha-21 (α_{21})	0.64 Antagonism	Undefined	1.07 Synergism	0.86 Antagonism
Gamma-12 (γ_{12})	0.63 Antagonism	Undefined	Undefined	5.39 Synergism
Gamma-21 (γ_{21})	1.67 Synergism	Undefined	10.2 Synergism	0 Antagonism

In the last years, there is a tendency to resort to drug combinations to treat chronic diseases with complex pathophysiology. Being a chronic pathology, diabetic neuropathy is difficult to treat, affecting the patients' quality of life. Thus, the discovery of new therapies with improved efficacy is of interest. In this regard, several preclinical studies have been identified in the specialized literature that evaluated the effect of different drug combinations in the relief of different types of diabetic neuropathy pain.

Regarding the efficacy measured by means of the beta indicator of the sildenafil-metformin combination, the cold hypersensitivity test showed a synergistic interaction between sildenafil and metformin, with a more pronounced effect 14 days after treatment administration. Regarding the clinical interpretation of the calculated values of the alpha indicator, for Case 3 (synergistic interaction), sildenafil and metformin mutually increased their potency in heat hypersensitivity. This may translate into a lower required dose of sildenafil in the presence of metformin, as well as

a lower required dose of metformin in the presence of sildenafil when the goal is to reduce pain hypersensitivity to heat.

7. ED50 prediction for sildenafil-metformin combination in an animal model of alloxan-induced diabetic neuropathy in rats

To maximize the intended benefits and reduce the risk of side effects, the individualization of the drug dose is often a challenge. The ED_{50} represents the dose at which the desired response occurs for 50% of the population and is calculated by drawing a line corresponding to the 50% effect on the dose-response curve. Using the dose that produces approximately 50% of the maximum possible effect of the drug has been shown to be sufficient to achieve the desired effect. It is necessary to point out that with the increase of the dose, the risk of side effects also increases [51]. In this study we decided to evaluate the ED_{50} efficacy of the sildenafil-metformin combination previously calculated in Chapter 5 in an animal model of diabetic neuropathy.

Materials and methods

The experimental procedures were carried out in accordance with the bioethical rules proposed by Law 43/2014 on the protection of animals used for scientific purposes and Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010, on the protection of animals used for scientific purposes. The experimental protocol was approved by the Bioethics Commission of the Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, Bucharest, Romania.

For this study we selected 70 diabetic rats and 10 non-diabetic rats divided into 8 equal groups (n=10) which received daily treatment, for 15 days by oral gavage, as follows: non-diabetic control group (ND) – distilled water 1 $mL \cdot kg^{-1}$; diabetic control group (D) - distilled water 1 $mL \cdot kg^{-1}$; G30 group-gabapentin 30 $mg \cdot kg^{-1}$; G90 group-gabapentin 90 $mg \cdot kg^{-1}$; G150 group-gabapentin 150 $mg \cdot kg^{-1}$; S2+M100 group-sildenafil 2 $mg \cdot kg^{-1}$ + metformin 100 $mg \cdot kg^{-1}$; S2.5+M300 group – sildenafil 2.5 $mg \cdot kg^{-1}$ + metformin 300 $mg \cdot kg^{-1}$; S3+M500 group-sildenafil 3 $mg \cdot kg^{-1}$ + metformin 500 $mg \cdot kg^{-1}$.

We evaluated blood sugar, thermal hypersensitivity to hot and cold stimulus, but also tactile hypersensitivity during 15 days of treatment. We also assessed the level of TNF-α, IL-6, NOS activity and thiol concentration in rat brain and liver homogenates.

Statistical analysis of the experimental data and their graphical illustration were performed using the GraphPad Prism v.5.00 software package (GraphPad Software, San Diego, CA, USA). The type of data distribution was determined using the D'Agostino-Pearson test. Experimental results were analyzed using the following statistical tests: one-way analysis of variance (ANOVA) test followed by Dunnet's post hoc test for parametric data and Kruskal-Wallis test followed by Dunn's post hoc test for nonparametric data.

Results and discussion

When talking about chronic treatments, the use of the lowest effective dose is preferable [51]. Manufacturers' studies for ED_{50} determination in some cases provide an estimate of ED50 and a range of ED_{50} in the population, but this information appears to have little influence on prescribing in some situations. Specialists in the field are either unaware of the importance of ED_{50} or completely ignore it. At the same time, the practical implications of the individual variations of ED_{50} depending on the pharmacokinetic and pharmacodynamic characteristics, but also on the body dimensions, are ignored [52].

The results of our research demonstrated a marked decrease in heat sensitivity for the group treated with the combination of ED_{50} of sildenafil and ED_{50} of metformin (group S2.5+M300) after 14 days of treatment in the Hot-plate test (Figure 7.1.A). Thus, this association registered a decrease in heat hypersensitivity induced by diabetic neuropathy comparable to that of the association between sildenafil and metformin that used higher doses than ED_{50}.

Also, increased pain reaction latency was also noted in the Cold-plate test. The S2.5+M300 and S3+M500 groups demonstrated significant decreases in pain sensitivity after 15 days of treatment when compared to the diabetic control group (Figure 7.1.B).

The antihyperalgesic effect was also noted in the tactile hypersensitivity test both for the sildenafil 2.5 $mg \cdot kg^{-1}$ + metformin 250 $mg \cdot kg^{-1}$ and sildenafil 3 $mg \cdot kg^{-1}$ + metformin 500 $mg \cdot kg^{-1}$ combinations after 14 days of treatment (Figure 7.1.C).

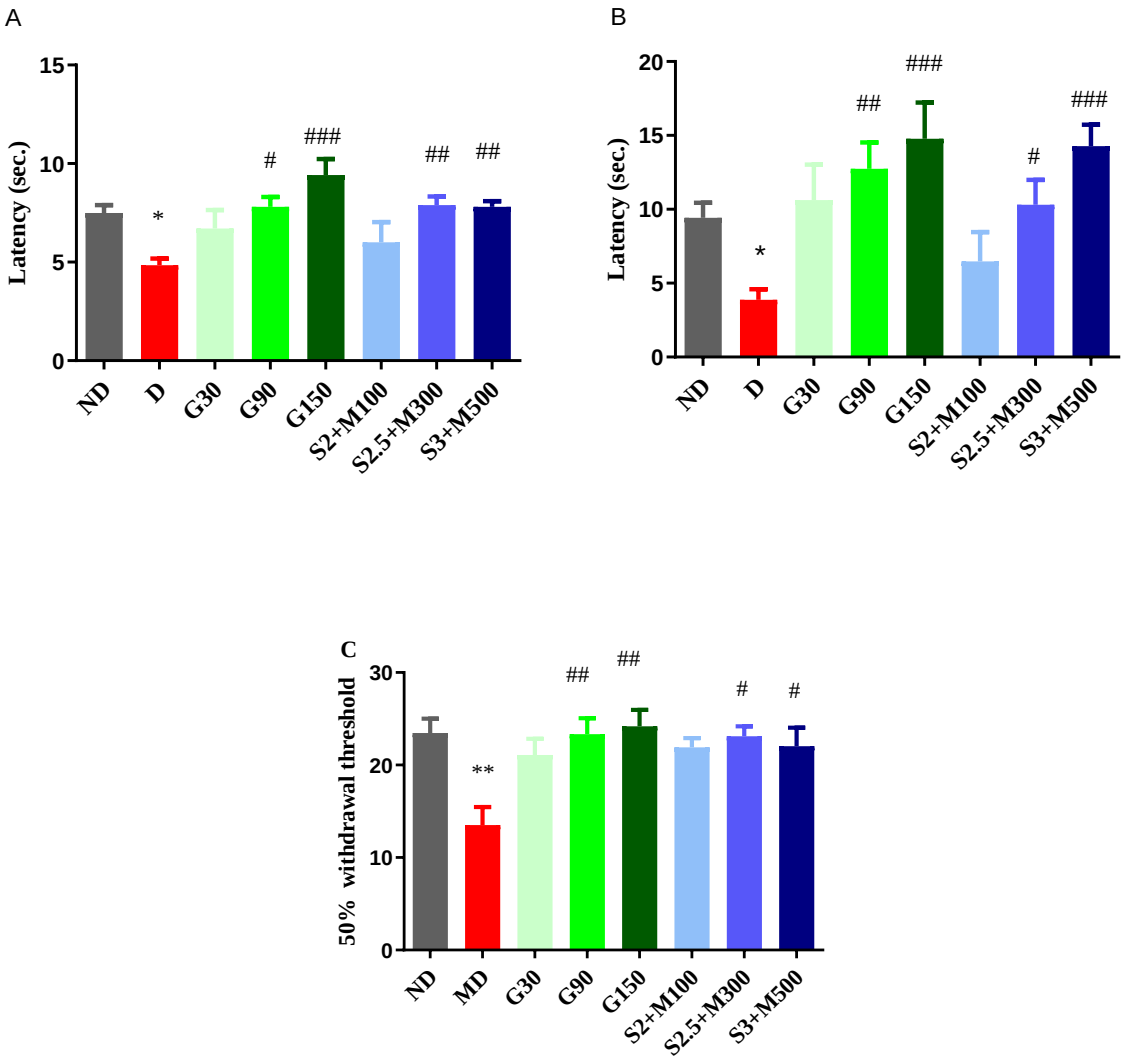

Figure 7.1. (A) Pain reaction latency in Hot-plate test after 14 days **(B)** Pain reaction latency in Cold-plate test after 15 days **(C)** 50% withdrawal threshold after 14 days

Values are expressed as mean ± S.E.M.

Also, our study revealed a decrease in TNF-α production following treatment with the sildenafil 2.5 mg·kg−1-metformin 250 mg·kg−1 and sildenafil 3 mg·kg−1 - metformin 500 mg·kg−1 combinations in the liver and the sildenafil 3 mg·kg−1- metformin 500 mg·kg−1 combination in brain tissues. In addition, the S2.5+M250 and S3+M500 groups had markedly reduced IL-6 levels when compared to the diabetic control group in both brain and liver tissue. Moreover, at the brain level, both sildenafil-metformin combinations recorded superior effects than the groups treated with gabapentin. The concentration of nitrites decreased after 15 days of

treatment with all 3 sildenafil-metformin combinations in both brain and liver tissues, and the concentration of total thiols increased for all sildenafil-metformin groups in both brain and liver tissues.

Conclusions and personal contributions

Considering the observations from previous studies, we evaluated in a first experiment the antihyperalgesic potential of sildenafil and metformin administered in 3 different doses in an animal model of diabetic neuropathy.

To induce diabetic neuropathy, we administered 2 different doses of alloxan - 130 mg·kg^{-1} and 150 mg·kg^{-1} to mice.

In the Hot-plate test, all doses of metformin (150 mg·kg^{-1}, 250 mg·kg^{-1}, 500 mg·kg^{-1}), sildenafil 2.5 mg·kg^{-1} and sildenafil 3 mg·kg^{-1} showed a significant increase in pain reaction latency after 14 days of treatment. In the tail withdrawal test, metformin administered in doses of 150 mg·kg^{-1} and 500 mg·kg^{-1} and sildenafil in doses of 1.5 mg·kg^{-1} and 3 mg·kg^{-1} demonstrated significant increases in pain reaction latency after 14 days of treatment.

Moreover, the blood count showed increased values for leukocytes and mean platelet volume and decreased values for erythrocytes, hemoglobin and hematocrit in the diabetic groups. Due to better glycemic control, metformin administered in doses of 250 mg·kg^{-1} and 500 mg·kg^{-1} resulted in significant decreases in leukocyte values and statistically insignificant decreases in erythrocyte, hemoglobin, and hematocrit values.

We further analyzed the antihyperalgesic effect of different sildenafil-metformin combinations previously administered individually in an animal model of diabetic neuropathy in mice.

An increase in pain reaction latencies was observed in the tail withdrawal test and in the Hot-plate test. In addition, all 3 sildenafil-metformin combinations showed similar or superior effects than gabapentin in reducing pain sensitivity.

Moreover, the sildenafil 2.5 mg·kg^{-1} and metformin 250 mg·kg^{-1} combination demonstrated the ability to reduce interleukin-6 production in brain and liver tissues.

Also, the groups treated with sildenafil 2.5 mg·kg^{-1} + metformin 250 mg·kg^{-1} and sildenafil 3 mg·kg^{-1} + metformin 500 mg·kg^{-1} prevented the increase in cerebral nitric oxide synthase

activity, while all 3 sildenafil-metformin combinations showed significantly reduced values of nitrites in liver tissues.

Based on the results obtained in Chapters 4 and 5 in the heat and cold hypersensitivity tests following the administration of the substances in monotherapy and in combination, we evaluated the synergism of the combination with the help of the MuSyC algorithm.

Our results showed a synergistic interaction between sildenafil and metformin in the cold hypersensitivity test. In the heat hypersensitivity test, sildenafil and metformin potentiated each other.

In a final experiment, we assessed the ability of the combination between ED_{50} of sildenafil and of metformin, calculated with the help of the results obtained in the first experiment to reduce pain in an alloxan-induced diabetic neuropathy model in rats.

Thus, sildenafil 2.5 mg·kg^{-1} + metformin 250 mg·kg^{-1} combination, administered over a period of 15 days, increased the pain reaction latency in the Hot-plate and Cold-plate tests, but also in the von-Frey test.

Also, the administration of this combination demonstrated its anti-inflammatory action by decreasing the level of tumor necrosis factor (TNF-α) in the liver tissue and interleukin-6 in the brain and liver. Furthermore, sildenafil 2.5 mg·kg−1 + metformin 250 mg·kg−1 combination prevented the increase in nitric oxide synthase activity and showed protection against glutathione depletion.

The novelty of this research is given by the hypothesis of the use of two substances that can be frequently prescribed in the therapy of diabetic patients for the treatment of glucose metabolism disorder (metformin) and for vascular complications induced by hyperglycemia (sildenafil, PDE5 inhibitor). There are no published studies in the literature investigating the efficacy of sildenafil in diabetic neuropathy, or the association between metformin and sildenafil.

Personal contributions in this PhD thesis can be considered the following:

- ✓ Induction and validation of the method of diabetic neuropathy in mice by administration of alloxan. Correlation of the data on the changes produced by the metabolic disturbances given by the increased level of glucose and fructosamine and the increase in diabetic thermal pain sensitivity in the alloxan diabetes model (**Chapter 4**);

- ✓ Investigating the effect of metformin and sildenafil administered as monotherapy on pain sensitivity in diabetic mice, in correlation with fructosamine concentration and blood count (leukocytes, erythrocytes, hemoglobin, hematocrit, mean platelet volume) (**Chapter 4**).
- ✓ Determination of the mean effective doses (ED_{50}) for sildenafil and metformin in the heat and cold hypersensitivity tests in the previously described animal model (**Chapter 4**);
- ✓ Demonstration of the antihyperalgesic effect of fixed-dose combinations between sildenafil and metformin in alloxan-induced diabetes in mice, correlated with the influence of this combinations on pro-inflammatory cytokines (TNF-α and IL-6) and nitric oxide synthase activity in brain and liver homogenates. These determinations are the first in the literature, as the sildenafil – metformin combination has not been studied before (**Chapter 5**);
- ✓ Evaluation of the synergism of the sildenafil and metformin combination based on the experimental results obtained by determining the parameters of pain sensitivity in diabetic mice, using the MuSyC algorithm (calculation of three types of synergism indicators: efficacy, potency and cooperativity) (**Chapter 6**);
- ✓ Evaluation of the antihyperalgesic potential of the sildenafil and metformin combination, administered in mean effective doses (previously calculated) in alloxan-induced diabetic neuropathy in rats (**Chapter 7**).
- ✓ Demonstration of the reduction of the pro-inflammatory cytokines IL6 and TNF-α release in the brain and liver tissue following the administration of sildenafil and metformin combinations in diabetic rats, along with the decrease in nitric oxide synthase activity and increase of the protection against glutathione depletion (**Chapter 7**).

Selective references

1. Pain - International Association for the Study of Pain (IASP). https://www.iasp-pain.org/publications/pain/ (accessed Sep. 06, 2022).
2. Rusbridge C, Jeffery ND. Pathophysiology and treatment of neuropathic pain associated with syringomyelia. *Vet J*, 175(2):164–172, 2008.
3. IDF Diabetes Atlas. https://idf.org/e-library/epidemiology-research/diabetes-atlas.html (accessed Mar. 07, 2023).
4. Davies M, Brophy S, Williams R, Taylor A. The Prevalence, Severity, and Impact of Painful Diabetic Peripheral Neuropathy in Type 2 Diabetes. *Diabetes Care*, 29(7):1518–1522, 2006.
5. Callaghan BC, Cheng HT, Stables CL, Smith AL, Feldman EL. Diabetic neuropathy: clinical manifestations and current treatments. *Lancet Neurol*, 11(6):521–534, 2012.

6. Peltier A, Goutman SA, Callaghan BC. Painful diabetic neuropathy. *BMJ*, 348, 2014.
7. Pop-Busui R, Boulton AJM, Feldman EL, *et al.* Diabetic Neuropathy: A Position Statement by the American Diabetes Association. *Diabetes Care*, 40(1):136–154, 2017.
8. Boulton AJM. Diabetic neuropathy and foot complications. *Handb Clin Neurol*, 126:97–107, 2014.
9. Bowling FL, Rashid ST, Boulton AJM. Preventing and treating foot complications associated with diabetes mellitus. *Nat Rev Endocrinol*, 11(10):606–616, 2015.
10. Tesfaye S, Chaturvedi N, Eaton SEM, *et al.* Vascular risk factors and diabetic neuropathy. *N Engl J Med*, 352(4):341–350, 2005.
11. Tesfaye S, Vileikyte L, Rayman G, *et al.* Painful diabetic peripheral neuropathy: consensus recommendations on diagnosis, assessment and management. *Diabetes Metab Res Rev*, 27(7):629–638, 2011.
12. Dunnigan SK, Ebadi H, Breiner A, *et al.* Conduction Slowing in Diabetic Sensorimotor Polyneuropathy. *Diabetes Care*, 36(11):3684, 2013.
13. Gumy LF, Bampton ETW, Tolkovsky AM. Hyperglycaemia inhibits Schwann cell proliferation and migration and restricts regeneration of axons and Schwann cells from adult murine DRG. *Mol Cell Neurosci*, 37(2):298–311, 2008.
14. Mizisin AP, Shelton GD, Wagner S, Rusbridge C, Powell HC. Myelin splitting, Schwann cell injury and demyelination in feline diabetic neuropathy. *Acta Neuropathol*, 95(2):171–174, 1998.
15. Dyck PJ, Overland CJ, Low PA, *et al.* Signs and symptoms versus nerve conduction studies to diagnose diabetic sensorimotor polyneuropathy: Cl vs. NPhys trial. *Muscle Nerve*, 42(2):157–164, 2010.
16. Feldman EL, Nave KA, Jensen TS, Bennett DLH. New Horizons in Diabetic Neuropathy: Mechanisms, Bioenergetics, and Pain. *Neuron*, 93(6):1296–1313, 2017.
17. Nowicki M, Kosacka J, Serke H, Blüher M, Spanel-Borowski K. Altered sciatic nerve fiber morphology and endoneural microvessels in mouse models relevant for obesity, peripheral diabetic polyneuropathy, and the metabolic syndrome. *J Neurosci Res*, 90(1):122–131, 2012.
18. Cavalli E, Mammana S, Nicoletti F, Bramanti P, Mazzon E. The neuropathic pain: An overview of the current treatment and future therapeutic approaches. *Int J Immunopathol Pharmacol*, 33, 2019.
19. Pușcașu C, Zanfirescu A, Negreș S. Recent Progress in Gels for Neuropathic Pain. *Gels*, 9(5):417, 2023.
20. Attal N. Pharmacological treatments of neuropathic pain: The latest recommendations. *Rev Neurol (Paris)*, 175(1–2):46–50, 2019.
21. Nehra A, Moreland RB. Neurologic erectile dysfunction. *Urol Clin North Am*, 28(2):289–308, 2001.
22. Gerber RE, Vita JA, Ganz P, *et al.* Association of Peripheral Microvascular Dysfunction and Erectile Dysfunction. *J Urol*, 193(2):612–617, 2015.
23. Thompson IM. Erectile Dysfunction and Subsequent Cardiovascular Disease. *JAMA*, 294(23):2996, 2005.
24. Selvin E, Burnett AL, Platz EA. Prevalence and Risk Factors for Erectile Dysfunction in the US. *Am J Med*, 120(2):151–157, 2007.
25. Corbin JD, Francis SH. Cyclic GMP Phosphodiesterase-5: Target of Sildenafil. *J Biol Chem*, 274(20):13729–13732, 1999.
26. Wang L, Chopp M, Szalad A, *et al.* Sildenafil ameliorates long term peripheral neuropathy in type II diabetic mice. *PLoS One*, 10(2), 2015.

27. Melemedjian OK, Mejia GL, Lepow TS, Zoph OK, Price TJ. Bidirectional regulation of P body formation mediated by eIF4F complex formation in sensory neurons. *Neurosci Lett*, 563:169–174, 2014.
28. Mao-Ying QL, Kavelaars A, Krukowski K, *et al.* The Anti-Diabetic Drug Metformin Protects against Chemotherapy-Induced Peripheral Neuropathy in a Mouse Model. *PLoS One*, 9(6):e100701, 2014.
29. Shiers S, Pradhan G, Mwirigi J, *et al.* Neuropathic Pain Creates an Enduring Prefrontal Cortex Dysfunction Corrected by the Type II Diabetic Drug Metformin But Not by Gabapentin. *J Neurosci*, 38(33):7337–7350, 2018.
30. Pușcașu [1] C, Mihai P, Zbârcea CE, *et al.* Investigation of antihyperalgesic effects of different doses of sildenafil and metformin in alloxan-induced diabetic neuropathy in mice. *Farmacia* 2, 71:3, 2023.
31. Yu L-N, Yang X-S, Hua Z, Xie W. [Serum levels of pro-inflammatory cytokines in diabetic patients with peripheral neuropathic pain and the correlation among them]. *Zhonghua Yi Xue Za Zhi*, 89(7):469–471, 2009.
32. Brown AS, Hong Y, De Belder A, *et al.* Megakaryocyte ploidy and platelet changes in human diabetes and atherosclerosis. *Arterioscler Thromb Vasc Biol* 1997; 17(4):802–807.
33. Eriksson U, Ewald U, Tuvemo T. Increased platelet volume in manifest diabetic rats. *Ups J Med Sci*, 88(1):17–23, 1983.
34. J. Price T, Das V, Dussor G. Adenosine Monophosphate-activated Protein Kinase (AMPK) Activators For the Prevention, Treatment and Potential Reversal of Pathological Pain. *Curr Drug Targets*, 17(8):908–920, 2016.
35. Byrne FM, Cheetham S, Vickers S, Chapman V. Characterisation of Pain Responses in the High Fat Diet/Streptozotocin Model of Diabetes and the Analgesic Effects of Antidiabetic Treatments. *J Diabetes Res*, 2015:1–13, 2015.
36. Deftu A-F, Chu Sin Chung P, Laedermann CJ, *et al.* The Antidiabetic Drug Metformin Regulates Voltage-Gated Sodium Channel Na V 1.7 via the Ubiquitin-Ligase NEDD4-2. *Eneuro*, 9(2):ENEURO.0409-21.2022, 2022.
37. Zhang H, Zhang H, Dougherty PM. Dynamic effects of TNF-α on synaptic transmission in mice over time following sciatic nerve chronic constriction injury. *J Neurophysiol*, 110(7):1663–1671, 2013.
38. Hills CE, Brunskill NJ. Cellular and physiological effects of C-peptide. *Clin Sci*, 116(7):565–574, 2009.
39. Phaniendra A, Jestadi DB, Periyasamy L. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. *Indian J Clin Biochem*, 30(1):11–26, 2015.
40. Levy D, Zochodne DW. NO Pain: Potential Roles of Nitric Oxide in Neuropathic Pain. *Pain Pract*, 4(1):11–18, 2004.
41. Kouidrat Y, Pizzol D, Cosco T, *et al.* High prevalence of erectile dysfunction in diabetes: a systematic review and meta-analysis of 145 studies. *Diabet Med*, 34(9):1185–1192, 2017.
42. Escobar-Jiménez F. Eficacia y seguridad del sildenafilo en varones con diabetes mellitus tipo 2 y disfunción eréctil. *Med Clin (Barc)*, 119(4):121–124, 2002.
43. Shah P, Trivedi N. A meta-analysis on efficacy and tolerability of sildenafil for erectile dysfunction in patients with diabetes mellitus. *Indian J Sex Transm Dis AIDS*, 39(1):1, 2018.
44. Buse JB, Wexler DJ, Tsapas A, *et al.* 2019 Update to: Management of Hyperglycemia in Type 2 Diabetes, 2018. A Consensus Report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetes Care*, 43(2):487–493, 2020.

45. Zhou X, Seto SW, Chang D, *et al*. Synergistic Effects of Chinese Herbal Medicine: A Comprehensive Review of Methodology and Current Research. *Front Pharmacol*, 7, 2016.
46. Devita VT, Young RC, Canellos GP. Combination versus single agent chemotherapy: A review of the basis for selection of drug treatment of cancer. *Cancer*, 35(1):98–110, 1975.
47. Chesney MA, Morin M, Sherr L. Adherence to HIV combination therapy. *Soc Sci Med*, 50(11):1599–1605, 2000.
48. Jukema JW, van der Hoorn JW. Amlodipine and atorvastatin in atherosclerosis: a review of the potential of combination therapy. *Expert Opin Pharmacother*, 5(2):459–468, 2004.
49. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med*, 17(11):1410–1422, 2011.
50. Wooten DJ, Albert R. synergy: a Python library for calculating, analyzing and visualizing drug combination synergy. Wren J, ed. *Bioinformatics*, 37(10):1473–1474, 2021.
51. Dimmitt S, Stampfer H, Martin JH. When less is more - efficacy with less toxicity at the ED50. *Br J Clin Pharmacol*, 83(7):1365–1368, 2017.
52. Cross J, Lee H, Westelinck A, Nelson J, Grudzinskas C, Peck C. Postmarketing drug dosage changes of 499 FDA-approved new molecular entities, 1980-1999. *Pharmacoepidemiol Drug Saf* , 11(6):439–446, 2002.

List of published articles:

1. **Pușcașu, C**; Zanfirescu, A; Negreș, S. Recent progress in gels for neuropathic pain. *Gels*, 9(5):417, 2023. https://doi.org/10.3390/gels9050417. FI = 4,432; Q1 (Chapter 1, pages 16, 35, 36).
2. **Pușcașu, C**; Mihai, DP; Zbârcea, CE; Zanfirescu, A; Chiriță, C; Ghiță, CIV; Negreș, S. Investigation of antyhiperalgesic effects of different doses of sildenafil and metformin in alloxan-induced diabetic neuropathy in mice. *Farmacia*, 3:71, 2023. https://doi.org/10.31925/farmacia.2023.3.19. FI = 1,62; Q2 (Chapter 4, pages 42-70).
3. **Pușcașu C**, Ungurianu A, Șeremet OC, Andrei C, Mihai DP, Negreș S. The Influence of Sildenafil–Metformin Combination on Hyperalgesia and Biochemical Markers in Diabetic Neuropathy in Mice. *Medicina*, 59(8):1375, 2023. https://doi.org/10.3390/medicina59081375. FI= 2,948; Q3 (Chapter 5, pages 71-93).