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„CAROL DAVILA", BUCUREŞTI
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The effect of treatment with SGLT2 inhibitors on gut microbiota and inflammatory status in patients with type 2 diabetes mellitus

PhD Supervisor:
PROF. GUJA CRISTIAN, MD, PhD

PhD Student:
MÎNDRESCU NICOLETA MIHAELA

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Summary

The 21st century brought about a better quality of life for humanity, through the technological revolution, increased connectivity and mobility, higher levels of education and, last but not least, precise, high-performance medicine. Modern society also comes, however, with certain disadvantages, such as the alarming increase in the prevalence of chronic diseases - cardiovascular diseases, cancer, obesity, and type 2 diabetes. Diabetes affects an estimated 589 million adults between the ages of 20 - 79 worldwide, as of 2024. 95% of all diabetes patients suffer from type 2 diabetes and its multiple related chronic complications, which brings this disease to pandemic-level proportions. Furthermore, its long-term care and treatment plans place an enormous financial strain on society.

Type 2 diabetes has been known since antiquity. Its central element is hyperglycemia, which, over time, harms affected organs through the development of chronic disabling micro- and macrovascular complications. Common examples are diabetic retinopathy, diabetic nephropathy, diabetic neuropathy, cardiovascular disease, cerebrovascular disease, and peripheral arterial disease. The etiology of diabetes is multifactorial and includes genetic risk factors, as well as environmental and lifestyle factors. Risk factors are categorized into (1) **non-modifiable** risk factors (such as family history, genetic predisposition, age, sex, race, weight at birth) and (2) **modifiable** risk factors (obesity, smoking, sedentary lifestyles, inadequate diets, sleep deprivation, etc.). Between 60-70% of type 2 diabetes patients have at least one diagnosed relative, which makes family history and genetic predispositions the most important **non-modifiable** factors. Obesity is the most important **modifiable** risk factor of diabetes, affecting over 90% of all patients.

The etiopathogenic mechanisms of type 2 diabetes are extremely complex and not yet fully understood. The onset and progression of the disease are triggered by several physiopathological modifications, coined by Ralph De Fronzo as “the Ominous Octet”, and involving multiple components: the skeletal muscle, the liver, the adipose tissue, α and β pancreatic cells, the gastrointestinal tract, the kidney and the brain. The main pathogenic mechanisms are **the alteration of insulin production** and **the increase of insulin resistance** in hepatic, muscular and adipose tissue. Pancreatic β cell dysfunction is a key cause of reduced insulin secretion in the pancreas and consecutive hyperglicemia. Insulin resistance

decreases the peripheral use of glucose and increases its production in the liver, unequally affecting the involved metabolic pathways.

A mechanism that recently caught the attention of the medical world is the involvement of the gut microbiota (GM) in the pathogeny of type 2 diabetes, through the so-called **dysbiosis.** Dysbiosis is defined as imbalances of the various populations of microorganisms (bacteria, viruses, protozoa, fungi) living in the digestive tract. The **Microbiota** and **microbiome** are two related concepts whose etymology stems from the Greek words *micros* (small) and *bios* (life). The microbiota represents the totality of microorganisms, while the microbiome (or microbial ecosystem) represents the entire habitat that includes the organisms listed above, the sum of their genes and the intestinal environment where these elements interact. The microbiome and the human genome form the metagenome.

The gut microbiota is an ecological system made up of approximately 100 trillion microorganisms, 10 times more than the number of cells in the human body. 90% of the microbiota consists of bacteria, and its total weight is approximately 1.5 kg, similar to the liver - the largest human organ. Its density and composition increase from top to bottom, reaching the greatest diversity in the colon. The bacterial microbiota is classified into 10 phyla, 1000 species and over 17000 subspecies. The intestine of any given individual only contains a tiny fraction of these subspecies, between 800-1200. The most prevalent phyla (over 70%) are *Bacteroidetes* and *Firmicutes*. The former consists of gram-negative bacteria (the best known of which is *Prevotella*), while the latter consists of gram-positive bacteria (e.g. *Clostridum, Eubacterium, Roseburia, Ruminococcus, Lactobacillus*). The relationship between these two populations, expressed as a *Firmicutes/Bacteroides* ratio, is associated with several pathological conditions, especially increased obesity. Several types of relationships are developed between microbial species: **positive** (mutualism, synergism, commensalism), **negative** (parasitism, antagonism) and **neutral.** Their constant interaction deeply influences both the dynamics and the functionality of the microbiome. Today, it is known that the microbiome - this true “virtual organ” of the human body - has over 3 million genes, orders of magnitude more than the human genome itself (23000 genes). The microbial composition of each individual is unique, and begins taking shape in the first days after birth. It depends on the gestational age at birth, the genetics of the host, the type of birth (natural, C-section, water), dietary diversification, the use of antibiotics and more. The microbiota

has a **stable** part (determined in the first years of life), and a **variable** part (dependent on diet, stress, medication etc.) Nutrition is extremely important for the regulation of the gut microbiota - the diet represents an information exchange between the environment and the human body. Ingested food, once digested and transformed into substrates, reaches the intestine, where it comes into contact with bacteria possessing genes that encode different enzymes involved in digestion. A typical Western diet rich in carbohydrates and fats leads to excessive proliferation of bacteria specialized in digesting these substrates - bacteria which are related to insulin resistance and inflammation.

Microorganisms living in the gastrointestinal tract are strongly involved in many physiological processes of the host, such as controlling inflammation and immune response, ensuring the integrity of the intestinal barrier, the metabolism of fatty acids and drugs, vitamin production, protein synthesis, energy extraction from certain otherwise indigestible substrates, intestinal motility, etc. An essential role in many of these processes is played by bacterial metabolites, such as short-chain **free fatty acids** (FFAs): acetate, butyrate, propionate. These are involved in decreasing intestinal inflammation, pathogenic invasion, activating immune cells and regulating the expression of pro-inflammatory cytokines (IL-6, IL-12, TNF-α).

In addition to its essential roles in human physiology, recent research has shown that the gut microbiota is deeply involved in the onset and progression of many chronic diseases, such as cardiovascular, neuropsychiatric, nephrological, gastroenterological, oncological, metabolic diseases and more. For example, the interaction of the cardiovascular system with the gut microbiota is based on inflammation, mediated by a series of molecules, some with a protective role (short-chain FFAs), others with an inverse effect (trimethylamine, bile acids, lipopolysaccharides). Intestinal dysbiosis is observed in neurodegenerative diseases, such as Alzheimer's disease, as inflammation and permeabilisation of the intestinal mucosa, followed by neuroinflammation, hyperphosphorylation and amyloid accumulation. Parkinson’s disease is significantly correlated with *Helicobacter pylori.* In children affected with autism spectrum disorders, the detected concentration of *Clostridium* was 10 times higher compared to the control group. In inflammatory bowel diseases, both microbial diversity and the population of *Firmicutes* decrease, while the population of *Enterobacter, Proteobacter* and *Candida albicans* increases. Nonalcoholic fatty liver is associated with a

decrease in some anti-inflammatory bacterial strains (*Ruminococcus, Faecalibacterium)* and the increase of pro-inflammatory ones (*Escherichia coli, Fusobacterium, Prevotella* etc).

Some bacterial strains are also involved in the development of malignant diseases, especially through toxic metabolites with a pro-inflammatory role. *Escherichia coli* (through its metabolite colibactin), certain species of the *Enterobacteriaceae* family and *Prevotella* are involved in the pathogenesis of colorectal cancer, while *Lactobacillus reuteri* has a protective role. In gastric cancer, *Helicobacter pylori* infection is responsible for 65-80% of diagnosed cases, according to statistics.

The etiopathogenesis of metabolic diseases is also associated with intestinal dysbiosis, in addition to environmental and environmental factors. A defining element of obesity is the abundance of *Firmicutes* and the decrease in the population of *Bacteroidetes* and *Bifidobacter*. There is already ample evidence from both animal and human studies that the gut microbiota plays an important role in type 2 diabetes mellitus. Dysbiosis associated with Type 2 diabetes is characterized by the increase in some species of pathogenic and opportunistic gram-negative bacteria (*Enterobacteriaceae, Clostridiales, Escherichia coli, Bacteroides caccae, Prevotella copri, Bacteroides vulgates)*, the reduction of microbial diversity, the increase of intestinal permeability, inflammation, metabolic endotoxemia and, secondarily, insulin resistance. According to human and animal studies, one bacteria with a significantly reduced population in type 2 diabetes and prediabetes is *Faecalibacterium prausnitzii*, a butyrate producer with anti-inflammatory properties. Another bacterium with a metabolic role is *Akkermansia muciniphila*, which is considered a biomarker of a healthy metabolic profile. Its population decreases with type 2 diabetes, obesity and atherosclerosis. The same downward trend is also observed for *Bifidobacterium* and *Lactobacillus* populations. All these different types of bacteria represent potent probiotics that will certainly find their place in type 2 diabetes therapy, alongside known normoglycemic medication, either as food additives or as functional foods.

The dysbiotic profile of type 2 diabetes is far from uniform, as demonstrated by metagenomics studies. This inter-individual variability correlates not only with the risk of disease onset and progression, but also with the response to nutritional and pharmacological interventions, bringing into question the need for Machine Learning methods that allow phenotyping.

One aspect of interest in recent years is the relationship between the gut microbiota and antihyperglyaemic medication, as it can significantly influence the efficacy of the treatment, its adverse reactions, as well as its beneficial pleiotropic effects. This opens up novel therapeutic strategies for this metabolic disease. On one hand, medication can influence the composition and function of the gut microbiota, and on the other hand, the latter can determine the individual's response to the treatment.

Metformin, from the biguanide family, is the longest-acting therapeutic agent in type 2 diabetes and also the most prescribed due to its availability, efficacy in lowering blood sugar, safety and affordable price. It acts at the intestinal level, where it modifies the composition of the gut microbiota, both in people with diabetes and healthy subjects. One argument to this point is the fact that in the jejunum, its concentration is 30-300 times higher than in plasma. Microbial changes after metformin administration result in the restoration of physiological microflora, with an increase in the population of *Enterobacteriales*, *Akkermansia muciniphila*, *Bifidobacterium*, *Lactobacillus plantarum*, *L.reuteri*, *L.gasseri* and a decrease in the population of pathogenic bacteria (*Roseburia*, *Dorea*, *Klebsiella*, *Clostridium*, *Intestinibacter bartlettii)*. The consequence is an increase in the concentration of short-chain FFA, the integrity of the gastrointestinal barrier, a decrease in lipopolysaccharides (LPS) and inflammation.

Acarbose, an alpha-glucosidase inhibitor, blocks intestinal glucose absorption by local action and lowers postprandial blood glucose, increasing the bioavailability of carbohydrates in the intestine. Its effect on the gut microbiota consists of increasing bacterial populations engaged in carbohydrate degradation, and increasing the concentration of short-chain FFA. The most important changes observed in animal and human studies are the increase in the populations of *Bifidobacterium*, *Lactobacillus* and *Ruminococcus,* the increase of the ratio between primary bile acids and secondary bile acids, and the decrease in the abundance of *Bacteroides*.

Incretin therapy is a modern type 2 diabetes medication. There are three therapeutic classes developed so far, namely: (1) dipeptidyl-peptidase-4 (DPP4) inhibitors, (2) GLP-1 agonists and (3) dual agonists of GLP-1 and GIP (glucose-dependent insulinotropic polypeptide). The relationship between the gut microbiota and incretin therapy is

bidirectional and complex. A series of microbial metabolites (LPS, indoles, secondary bile acids) stimulate the secretion of GLP-1. In turn, incretin therapy acts through multiple mechanisms, modulating the structure and function of the gut microbiota. **DPP-4 inhibitors** increase the population of *Lactobacillus* and *Turicibacter* and decrease that of *Bacteroidetes*. **GLP-1 agonists** improve dysbiosis by increasing the *Bacteroidetes* to *Firmicutes* ratio, increasing the abundance of *Akkermansia muciniphila* strains and bacterial diversity. This evidence is gathered from both animal and human studies. The result of the proliferation of beneficial strains is the increase in the production of short-chain FFA, which plays a role in immunity and in maintaining the integrity of the intestinal barrier, also stimulating the endogenous secretion of GLP-1. Tirzepatide, a **dual GLP-1 and GIP agonist**, has shown effects on modulating gut microbiota and bile acid metabolites, with increased *Akkermansia muciniphila* concentration and decreased cholic, chenodeoxycholic and lithocholic acid levels. In animal models, tirzepatide has been shown to improve dysbiosis, by increasing the concentrations of *Bacteroides, Proteobacter, Actinobacterium, Bifidobacterium, Clostridium* and *Akkermansia muciniphila* and decreasing the *Firmicutes* population.

SGLT2 inhibitors are a class of normoglycemic drugs that are considered game-changers in the treatment of type 2 diabetes. The efficiency of these molecules, the weight and blood pressure reductions they cause, their significant cardio-renal benefits and numerous pleiotropic effects are only partially explained by the renal mechanism of action, which is insulin-independent. There are already numerous studies suggesting the involvement of the gut microbiota in mediating these effects. The first data supporting this claim comes from studies on animal models, where dapagliflozin caused an increase in *Ruminococcus* strains, an increase in *Bacteroidetes* and a decrease in *Firmicutes*, leading to anti-inflammatory and antioxidant effects. Another study conducted in mice with diabetes and nephropathy showed that administering empagliflozin restores bacterial diversity, decreases the concentration of *Oscillibacter* (an LPS producer), increases the concentration of *Bacteroides* and the function of the occludin. In human subjects, empagliflozin increased beneficial bacteria (*Roseburia, Faecalibacterium, Eubacterium*) and reduced pathogenic strains (*Escherichia, Shigella, Hungatella, Bilophila*). All this data opens new perspectives in the treatment of type 2 diabetes. Identifying the individual characteristics of each patient's gut microbiota will enable a personalized treatment that achieves all therapeutic targets and prevents chronic complications.

In addition, **SGLT2 inhibitors** have also shown systemic and local, organ-specific anti-inflammatory effects. These are based on several mechanisms: decreased oxidative stress, improvement of mitochondrial dysfunction, decreased inflammasome activity, increased intracellular calcium and sodium levels, and reduction of macrophage recruitment. These effects are expressed through the decrease in pro-inflammatory markers (IL-6, PCR, TNF-α). Results from cardiovascular and renal studies provide additional evidence related to the reduction of inflammation at the endothelial, cardiac, renal, hepatic and adipose levels, where the population of pro-inflammatory M1 macrophages decreases and the population of anti-inflammatory M2 increases.

There is very little evidence suggesting the interaction between **Sulfonylureas** and **glinides** and the gut microbiota. **Thiazolidinediones** (pioglitazone), molecules with a role in decreasing insulin resistance in the adipose tissue, muscle, and liver, are no longer widely used in clinical practice, and thus not considered of interest in this regard.

The increasing incidence and prevalence of type 2 diabetes mellitus necessitates the introduction of new therapeutic strategies for both the prevention and treatment of this chronic disease. The new etiopathogenic links that bring the intestinal microbiome and its changes into the spotlight raise the issue of using pre- and probiotics as adjuvants. These have a role in improving gastrointestinal inflammation and dysbiosis, in increasing incretin hormones, and in decreasing insulin resistance and blood sugar. In addition to adopting a Mediterranean-type diet (which provides clear long-term benefits) and regular physical exercise, clinical studies have shown that **probiotic** supplementation can bring quantifiable improvements in the composition of the gut microbiota. Implicitly, this also improves the metabolic status of patients with type 2 diabetes mellitus. Increasing the intake of **prebiotics** such as fibers and oligosaccharides can have a similar effect.

Justifying the research topic

Choosing this research topic is justified by the need to expand the available knowledge on the complex interactions between the gut microbiota and antihyperglycemic medication. Type 2 diabetes is no longer seen as an isolated disorder of glucose metabolism, but as a condition characterized by chronic inflammation, endothelial dysfunction, hormonal imbalances and alterations in the intestinal microbiome. The literature clearly shows that

insulin resistance, one of the cornerstones of type 2 diabetes, is closely linked to chronic low-intensity inflammatory processes, determined by multiple causes including intestinal dysbiosis. From a therapeutic point of view, recent years have brought forth a class of innovative molecules, namely SGLT2 inhibitors, which are known not only for their glycemic reduction but also for their extraglycemic effects. Recent studies have shown that they can influence the composition of the gut microbiota, increasing beneficial populations (*Bifidobacter*, *Lactobacillus*, *Faecalibacter prausnitzii*) while reducing proinflammatory opportunistic bacteria. In addition, SGLT2 inhibitors significantly reduce inflammatory markers (CRP, IL-6, TNF-α), partially explaining the cardiovascular and renal benefits observed in large clinical trials. However, data is still limited, and while many studies are being conducted on both animal models or small populations, their results are not always consistent.

This research topic is also justified by the lack of consistent data from studies conducted on the local population of Romania, which has its genetic, dietary and socio-economic particularities. The proposed research aims to fill these gaps, using a well-characterized clinical and paraclinical cohort, subject to a clear therapeutic protocol (randomization between two arms: metformin + SGLT2 inhibitors versus metformin + DPP4i). The detailed, modern analysis of the gut microbiota and of the correlation between the results, inflammatory markers and metabolic parameters bring an added value to the research.

Another important motivation is the practical nature and direct applicability of the results. Understanding how SGLT2 inhibitors influence the gut microbiota and inflammation could allow for the personalization of treatment in patients with type 2 diabetes, the optimization of therapeutic regimens and even the development of combined strategies set to maximize metabolic benefits (association with pre/probiotics or dietary interventions).

Last but not least, the topic is relevant from a fundamental research perspective, attempting to elucidate pathophysiological mechanisms that are still incompletely understood. In a global context where personalized medicine and integrated approaches are gaining ground, this research aims to bridge modern pharmacology, microbiology, and inflammation, opening new directions for investigation and therapeutic intervention.

General and specific objectives

The general objective of this doctoral thesis is to investigate how treatment with SGLT2 inhibitors influences the gut microbiota and inflammatory status of patients with type 2 diabetes. The choice of this objective derives from the need to better understand the extraglycemic effects of new therapies, effects that may contribute to reducing cardiovascular and renal risk. Through a comprehensive analysis of the effect of SGLT2 inhibitors on metabolic, inflammatory and microbial parameters, the work aims to explore qualitative changes in bacterial diversity, but also quantitative changes in beneficial and pathogenic populations. In parallel, it also evaluates related changes in inflammatory markers.

Another general objective is to evaluate the correlations between changes in the gut microbiota and the evolution of metabolic parameters, to determine whether there is a cause-effect relationship between the two. Through this integrative approach, the research aims to contribute to the foundation of personalized medicine in diabetes, providing the clinician with additional information to adapt the treatment to the individual needs of the patient. A further general objective is the creation of a local database which reflects the particularities of Romanian patients with type 2 diabetes. This is motivated by the fact that most of the data available in the literature comes from international studies, conducted on populations with significant genetic, socio-economic, dietary differences.

The specific objectives consist of analyzing the changes in the gut microbiota before and after initiating treatment with iSGLT2. Using high-resolution molecular techniques, we monitor bacterial diversity, the abundance of beneficial species and the reduction of opportunistic ones. A second specific objective is analyzing changes in inflammatory markers, before and after 3 months of treatment with SGLT2 inhibitors. At the same time, their potential anti-inflammatory effect is evaluated, independent of glycemia, by measuring PCR, hs-PCR and IL-6. The third specific objective is to compare the evolution of metabolic parameters between two groups of patients (one treated with metformin and iSGLT2, and the other with metformin and iDPP4), in order to detect possible differences in metabolic, anti-inflammatory and microbial efficacy. The fourth specific objective aims to identify, by leveraging multivariate statistical models, some factors influencing the therapeutic response, such as age, sex, BMI, abdominal circumference etc. An important specific objective is to

explore the correlation or causality between changes in the gut microbiota and changes in metabolic and inflammatory parameters. The final specific objective is to create an integrated database containing clinical, paraclinical, biochemical and microbiological information of the studied patients, for a more detailed analysis of the results. This has the potential to also serve as a starting point for future projects, multicenter research or meta-analyses.

Research hypotheses

To help build a clear picture of the effects of SGLT2 inhibitor treatment on the gut microbiota and inflammatory status of patients with type 2 diabetes, we formulated **six main hypotheses**.

Hypothesis 1: Treatment with iSGLT2 significantly increases the abundance of beneficial bacterial species (producers of short-chain free fatty acids) in patients with type 2 diabetes.

This hypothesis is based on current evidence, obtained mainly from animal model trials, which shows the increase of *Bifidobacter*, *Lactobacillus*, *Faecalibacter prausnitzii* after administration of iSGLT2. The hypothesis will be tested by analyzing the gut microbiota composition before and after 3 months of empagliflozin treatment. Statistical analysis will include paired difference tests (paired t-test or Wilcoxon) and the evaluation of alpha and beta diversity. The results will be interpreted in relation to the control group (patients treated with metformin + iDPP4).

Hypothesis 2: Treatment with iSGLT2 significantly reduces the abundance of pathogenic bacterial species (*Escherichia, Klebsiella* etc.) in patients with type 2 diabetes. This hypothesis will be tested using the same approach as Hypothesis 1, using identical statistical methods.

Hypothesis 3: Treatment with iSGLT2 reduces systemic inflammatory markers, independent of glycemic control.

The hypothesis is based on data from international trials showing that iSGLT2 have anti-inflammatory effects that are not exclusively dependent on lowering blood glucose. To test this, we will compare the values of inflammatory markers at baseline and at the final

visit, using parametric or non-parametric tests, depending on the distribution of the data. In addition, we will use regression models to control the effects of blood glucose on inflammation, assessing whether the reduction in inflammation is maintained after adjusting for HbA1c.

Hypothesis 4: **Treatment with iSGLT2 reduces the incidence of pathogenic intestinal fungi in patients with type 2 diabetes compared with DPP-4 inhibitors**.

Hypothesis 4 is based on the premise that treatment with iSGLT2 (empagliflozin) has beneficial effects not only on metabolic parameters, but also on the composition of the intestinal microbiota, in particular by reducing pathogenic fungal species (*Candida albicans*). The hypothesis is based on previous observations suggesting that treatment with empagliflozin may limit the proliferation of glycophilic fungi by inducing a more competitive and less permissive environment, secondary to bacterial remodeling (indirect antifungal effect).

Hypothesis 5: The effects of SGLT2 treatment on microbiota are more pronounced than those of DPP-4 inhibitors.

This hypothesis is based on the premise that the treatment with SGLT2 inhibitors has a deeper and broader impact on microbiota composition than the treatment with DPP-4 inhibitors.To evaluate this hypothesis, we compared the evolution of bacterial abundance between the two groups of patients. We analyzed the individual differences for 4 representative bacterial species-*Bifidobacterium, Faecalibacterium prausnitzii, Roseburia, Escherichia coli.*The statistical analysis included Wilcoxon tests for pairwise comparisons and Mann-Whitney test for differences between groups.

Hypothesis 6: Changes in the microbiota correlate with improvements in metabolic and inflammatory parameters, suggesting a mechanistic role of the microbiota in the benefits of iSGLT2 treatment.

This hypothesis aims to investigate whether the observed changes at the microbial level are just a side effect of the treatment or whether they correlate with metabolic and anti-inflammatory improvements. To test this, we will perform correlation analyses between bacterial changes (e.g. *Bifidobacterium* growth) and changes in glycemia, HbA1c, CRP, IL-6, as well as multivariate regression analyses to identify independent predictors of clinical improvement.

Materials and methods

The **materials and methods** of the trial consist of the enrolled population, the design of the research and the analysis methods that were employed. The group studied in this research consisted of 61 adult patients diagnosed with type 2 diabetes mellitus, recruited from the outpatient clinic of the "Prof. Dr. N. Paulescu" National Institute and other collaborating diabetes centers, between January 2023 and January 2024. These patients were carefully selected from a larger initial cohort of 72 pre-evaluated patients. They were included in the final database after applying the eligibility protocols and obtaining their informed consent.

Demographically, the final group included 30 men (49.2%) and 31 women (50.8%), with a mean age of 64.2 years, with a minimum of 37 years and a maximum of 75 years, representing an age group where diabetes is frequently diagnosed and treated. The majority (82%) came from urban areas. The mean duration of diabetes' evolution was approximately 5.5 years, varying between cases with a recent diagnosis and cases with older forms of the disease, marked by increasing use of medication. At the anthropometric level, the patients presented mean BMI values of 29.7 kg/m^2 (median value = 28.5, minimum value = 20.2, maximum value = 42.2). This places the majority of patients (41 out of 61) in the grade I or grade II obesity categories, according to the criteria of the World Health Organization. Abdominal circumference, an essential indicator for the assessment of central obesity and cardio-metabolic risk, had average values above the recognized risk thresholds, reinforcing the image of a population with increased metabolic risk.

In addition to the anthropometric profile, patients were also evaluated biochemically. The average values of glycated hemoglobin were 6.56%, with a minimum of 6 and a maximum of 9%. These indicate a moderate level of glycemic control, despite the use of metformin at an average dose of 2000 mg/day in men and 1500 mg/day in women. Fasting blood glucose values were also monitored, with an average of 129.38 mg/dL, along with a lipid profile characterized by total cholesterol of 174.63 mg/dL, LDL of 100.9 mg/dL, HDL of 50 mg/dL and triglycerides of 167.6 mg/dL. In addition, uric acid had mean values of 5.15 mg/dl, with a minimum of 2.2mg/dl and a maximum of 9.6mg/dl, suggesting an associated risk of hyperuricemia.

In terms of patients' inflammatory status, mean CRP-hs was 2.52 mg/L and mean IL-6 was 3.46 pg/mL, both marking a state of chronic low-intensity inflammation, specific to type 2 diabetes. This data is relevant because it allows assessing the impact of the therapeutic intervention not only on glycemic parameters, but also on systemic inflammation and changes in the intestinal microbiota.

Patients were divided into two distinct randomized therapeutic groups: a group treated with metformin and a DPP4 inhibitor (sitagliptin) and a group treated with metformin and a SGLT2 inhibitor (empagliflozin).

The **inclusion criteria** were rigorously established: age between 18 and 80 years, a type 2 diabetes diagnostic matching the diagnostic criteria established by the American Diabetes Association (ADA), glycated hemoglobin $\leq$ 9%, BMI > 20 kg/m^2. Another important criterion was the prior use of metformin as background therapy, which is considered the standard of initial treatment in type 2 diabetes. All patients agreed to voluntarily participate in the trial and also signed an informed consent, in conformance with international ethical norms.

The **exclusion criteria** were designed to minimize confounding variables and reduce the risk of adverse events that could compromise the integrity of the trial. Thus, patients diagnosed with type 1 diabetes mellitus were excluded, as the pathogenic mechanisms and treatment responses are fundamentally different from those in type 2 diabetes.

Patients with severe renal insufficiency, defined by an eGFR < 20 ml/min/1.73 m², were excluded, as this condition limits the use of SGLT2 inhibitors and could introduce additional risk factors. Patients with chronic gastrointestinal diseases (irritable bowel syndrome, celiac disease, inflammatory bowel diseases) were also excluded, as these conditions can directly influence the composition of the intestinal microbiota and the values of inflammatory markers, potentially distorting the results of the trial.

A further exclusion criterion was recent antibiotic use (within the last 30 days), which can profoundly alter the structure and function of the gut microbiota with persistent effects. Patients with active malignancies or other chronic inflammatory diseases, which can induce systemic changes in the inflammatory status, were also excluded to avoid bias.

Pregnant women and those planning a pregnancy during the trial period were excluded, both for safety reasons and because of the physiological and metabolic changes associated with pregnancy, which may influence the variables we analyze. Patients with known allergies to SGLT2 inhibitors or DPP4 inhibitors, or who were already enrolled in other clinical trials, were also excluded to avoid adverse reactions and overlap.

The **study design** was structured in several stages, from patient recruitment and application of eligibility criteria, to their randomization into treatment groups and longitudinal monitoring. The study was a randomized, prospective study, with assessments performed at two essential times: the initial visit (baseline) and the final visit, performed after three months of treatment. Patients were clinically and paraclinically evaluated at each of these times, and data was collected on anthropometric parameters, metabolic markers, gut microbiota composition and systemic inflammatory markers. An innovative aspect of the study was the fecal sampling and detailed analysis of the intestinal microbiome using culture methods.

Patients were randomized in a 1:1 ratio, using a computer-generated list, so that each eligible patient was randomly assigned to either the metformin + iSGLT2 treatment group or the metformin + iDPP4 (control) treatment group. This method allowed obtaining two groups comparable in terms of basic characteristics (age, sex, BMI, duration of diabetes), reducing the influence of confounding factors. In this study, we opted to use *minimization*, a dynamic and adaptive randomization method. The chosen minimization function was parameterized by two factors: the patient's age and the measured value of HbA1c. To ensure uniformity, patients were instructed to maintain a steady diet and physical activity level throughout the trial, so that the observed changes could mainly be attributed to the pharmacological treatment. The duration of the intervention in this trial was three months (12 weeks). This was chosen based on data from specialized literature, which shows that changes in the intestinal microbiota and inflammatory status can become detectable within this timeframe, even if certain metabolic effects occur more quickly. During the intervention, patients were monitored via phone calls and intermediate visits, to assess adherence to treatment and possible adverse effects. Recommendations were also provided regarding hydration, personal hygiene and recognition of symptoms of possible genitourinary infections, which is a known adverse effect of SGLT2 inhibitors.

An essential aspect of the design was also the respect for ethical principles: all patients were fully informed about the objectives and procedures of the study and signed an informed consent form. The study was approved by the ethics committee and was conducted in accordance with the Declaration of Helsinki.

The assessment of the gut microbiota was a central element of this study, with the role of exploring the influence of SGLT2 inhibitors treatment over the intestinal microbial ecosystem in patients with type 2 diabetes. To achieve an accurate analysis, the collection of fecal samples was performed under standardized conditions, according to international working protocols. Patients were instructed to collect the samples in sterile containers, specifically designed for the transport of biological material, in order to avoid external contamination and preserve the integrity of the samples. The collections were preferably performed in the early hours of the morning, and the samples were transported under standardized conditions to the central laboratory in Berlin (DIN EN ISO15189), where they were analyzed using the culture method. The culture media were used in accordance with European legislation, and were produced by Biomerieux and Becton Dickinson. The incubation time is 1-4 days, at a temperature of 30-35 degrees Celsius, depending on the bacterial or fungal strains to be detected. For identification and quantification, colonies of certain morphologies are measured in order to obtain a result in the format of CFU (colony forming units) per gram of fecal matter. Additional differentiation, when required, was obtained using the VITEK system, developed by Biomerieux. The evaluated parameters were microbial diversity, pH and composition in the main phyla, with the main goal of tracking the increase of beneficial bacteria and the reduction of pro-inflammatory bacteria.

In terms of the inflammatory profile, three main inflammatory markers were analyzed in the study: high-sensitivity C-reactive protein, interleukin 6 and standard C-reactive protein. These markers were assessed at two key time points: before treatment initiation (baseline) and after three months of therapeutic intervention, to capture the dynamics of therapy-induced changes. Comparing these values between the group treated with iSGLT2 + metformin and the control group (metformin + DPP4 inhibitor) allowed the identification of specific effects of SGLT2i on systemic inflammation.

These tests and analyses, together with the standard ones (complete blood count, lipid profile, urea, creatinine, uric acid etc.) were performed in Synevo laboratories, using calibrated equipment and internal and external quality controls. The samples were analyzed twice to minimize measurement errors, and the results were validated by specialized personnel with experience in biochemical and immunological diagnostics.

An important aspect in the description of the groups is the fact that patients treated with iSGLT2 experienced not only the reduction of blood glucose, but also beneficial side effects on body weight, blood pressure and on the intestinal microbiota. The treatment, while effective from a glycemic point of view, did not have the same pleiotropic effects on the control group, which highlights the differences between the two treatment plans.

The **statistical analysis** was carried out in several stages: (1) preparing the data, (2) descriptive analysis, (3) testing the differences between groups and changes before and after treatment, (4) analysis of the correlations and regressions, and (5) interpretation of the results in the context of the formulated hypotheses. Each stage was carried out carefully, respecting good statistical practices, to guarantee the quality and validity of the conclusions. The data was collected and recorded in the final database, subsequently analyzed using specialized statistical software (SPSS, Minitab, Excel). The applied tests included both descriptive analyses (means, standard deviations, confidence intervals), and comparative tests (t-test, Mann-Whitney U, ANOVA), Pearson/Spearman correlations and multivariate regression models. The significance threshold was set at $p < 0.05$.

RESULTS-General characteristics of patients

The analysis of the general characteristics of the patients included in the study is a crucial point for the accurate interpretation of the results. This allows the comparability of groups to be established and provides a solid framework for assessing the impact of therapeutic interventions on the markers studied. In terms of **gender distribution**, the patients were almost equally divided, with 31 women (50.8%) and 30 men (49.2%), ensuring the homogeneity of the sample. **The age** of the patients ranged from 37 to 75 years, with a mean of 64.25 ± 9.64 years and a median of 68 years. This reflects the typical demographic pattern of the type 2 diabetes population, with the majority of patients being middle-aged and

elderly. In terms of BMI, the mean value was 29.7 ± 4.81 kg/m², with a median of 28.5 kg/m², placing most patients in the overweight and obese categories**. Waist circumference**, a relevant marker of central obesity and metabolic risk, had a mean of 104.44 ± 12.15 cm, with a median of 103 cm; the maximum value recorded was 136 cm.

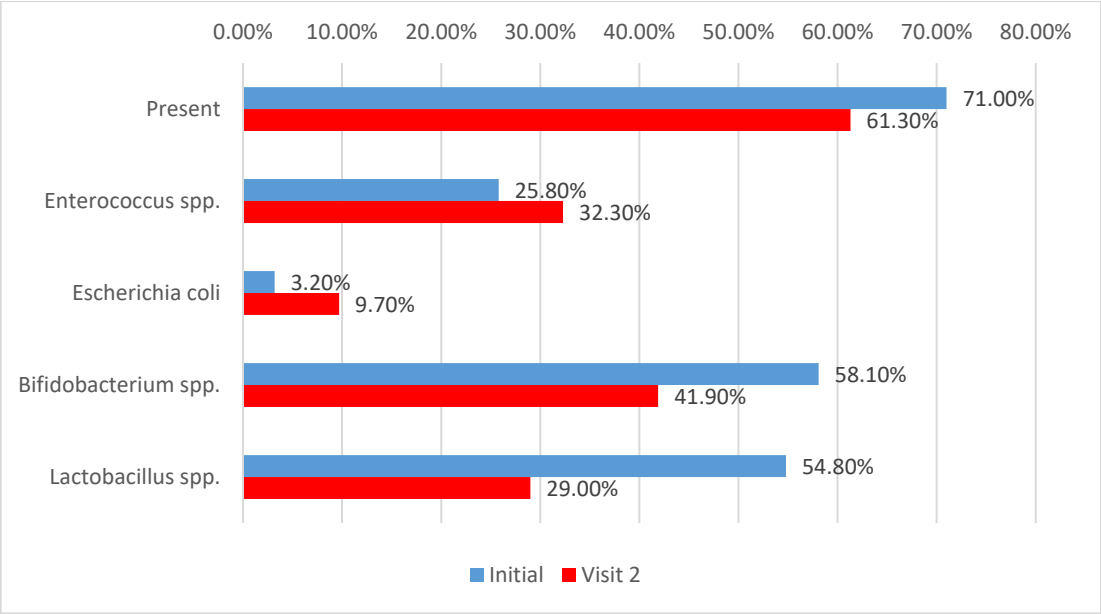
The initial biochemical profile of the patients in the study is detailed above. It reflects the classic patterns of the type 2 diabetes population: moderate glycemic control, frequent atherogenic dyslipidemia, generally conserved renal function with tendencies towards hyperuricemia, normal hematological values, as well as a chronic low-intensity inflammatory status. All these characteristics define a vulnerable metabolic terrain, in which persistent inflammation, lipid disorders, and pro-thrombotic risk combine and can amplify the cardiovascular and renal risks specific to the diabetic patient.

HYPOTHESIS TESTING

Hypothesis 1: Treatment with iSGLT2 significantly increases the abundance of beneficial bacterial species (producers of short-chain free fatty acids) in patients with type 2 diabetes compared to DPP4 inhibitors

According to the results, neither the frequency nor the type of low bacteria changed significantly over time ($p > 0.05$) in patients treated with Jardiance, except for the species *Lactobacillus spp.* ($p=0.021$), where a significant decrease in the reduced frequency of this bacterium was observed from the first visit to the second visit (54.8% vs. 29%).

Statistical analysis included paired t-test or Wilcoxon, depending on data distribution) to compare species abundance before and after treatment within the iSGLT2 group, assessment of alpha (Shannon and Simpson index) and beta (Bray-Curtis and Unifrac distances) microbial diversity, as well as intergroup comparisons between iSGLT2 and iDPP4 by independent tests (Mann–Whitney U and t-test). A comparative analysis of changes in the prevalence of bacterial species in the IDPP4 group revealed a general trend of improvement in the microbial profile. However, it did not reach statistical significance.



Evolving comparison of the existence and type of low bacteria in patients treated with Jardiance

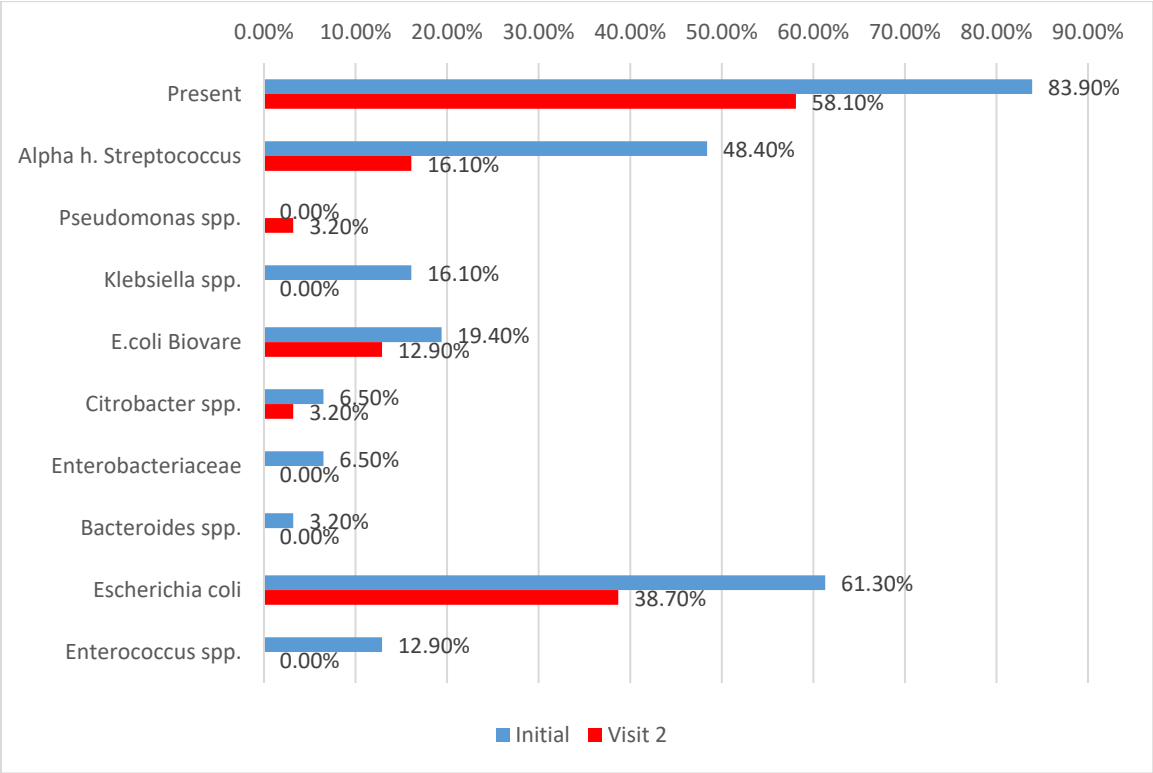
The hypothesis is only confirmed in the case of Lactobacillus.

Hypothesis 2: Treatment with iSGLT2 significantly reduces the abundance of pathogenic bacterial species (*Escherichia, Klebsiella,* etc.) in patients with type 2 diabetes mellitus

According to the results, the frequency of increased bacteria (83.9% vs. 58.1%, p = 0.039) and alpha-hemolytic Streptococcus species (48.4% vs. 16.1%, p = 0.013) decreased significantly at the second visit compared to the first visit, with a tendency towards a significant decrease in the frequency of *Klebsiella spp. Species.* (p=0.063) and *Escherichia coli* (p=0.065).

Analysis of diversity indices (Shannon, Simpson) revealed no statistically significant changes between the baseline and end points, suggesting that while trends in microbiota balancing exist, they do not reach robust significance thresholds over the analyzed period.

According to the results, neither the frequency nor the type of bacteria changed significantly over time (p > 0.05) in patients treated with Januvia.



Evolving comparison of the existence and type of bacteria grown in patients treated with Jardiance

Hypothesis 2 is confirmed.

Hypothesis 3: Treatment with iSGLT2 reduces levels of systemic inflammatory markers, independent of glycemic control

After three months of empagliflozin treatment, patients in the Jardiance group experienced a significant reduction in all three inflammatory markers analyzed: C-reactive protein, ultra-sensitive C-reactive protein, and interleukin-6. Specifically, mean CRP values decreased from 3.31 mg/L to 1.94 mg/L (p = 0.037), hs CRP from 2.44 mg/L to 1.52 mg/L (p = 0.042), and IL-6 from 3.72 pg/mL to 2.58 pg/mL (p = 0.040). These changes were statistically significant and remained relevant even after adjusting for HbA1c values, indicating that empagliflozin exerts an anti-inflammatory effect independent of improving glycaemic control. In contrast, in the sitagliptin-treated group, there were no significant changes in inflammatory markers. CRP values increased slightly, from 2.1 to 2.46 mg/L (p = 0.650), hs-CRP suffered a negligible increase (p = 0.485), and IL-6 levels remained virtually unchanged (p = 0.517). Multiple regression analysis revealed that the inflammation-reducing effect observed in the empagliflozin group is not explained by variations in HbA1c, suggesting a possible direct or pleiotropic effect of iSGLT2 on systemic inflammatory mechanisms.

The hypothesis is confirmed.

Hypothesis 4: Treatment with iSGLT2 reduces the incidence of intestinal pathogenic fungi in patients with type 2 DM compared to DPP-4 inhibitors

In the empagliflozin-treated group, after three months of therapy, notable changes in the intestinal fungal profile were observed. The proportion of patients with detectable fungi in the stool decreased significantly, from 46.7% to 23.3% (p = 0.031), suggesting a possible indirect antifungal effect of the treatment. A remarkable aspect was the complete disappearance of Candida albicans in three patients who had it at the time of baseline.

Fungal species	Prevalence of Visit 1 – N (%)	Prevalence Visit 2 – N (%)	Test McNemar – p-value
Total fungus	14 (46,7%)	7 (23,3%)	0.031
Candida albicans	8 (26,7%)	5 (16,7%)	0.157
Saccharomyces cerevisiae	3 (10%)	4 (13,3%)	0.625

Fungal Evolution in Jardiance Patients (Visit 1 vs Visit 2, Mc Nemar test

The results indicate a significantly greater decrease in the total prevalence of fungi in the Jardiance group, with a Δ of –23.4% compared to –3.3% in the Januvia group, a statistically significant difference ($p = 0.041$). For Candida albicans, the prevalence decreased by 10% in the iSGLT2 group, while in the DPP-4 group, there was no variation; however, this difference did not reach the threshold of statistical significance ($p = 0.226$).

The hypothesis is confirmed.

Hypothesis 5: The effects of iSGLT2 treatment on the microbiota are more pronounced than those of iDPP4 treatment

Bacterial species	Δ iSGLT2 (medium ± SD)	Δ iDPP4 (Medium ± SD)	p (Mann–Whitney U)
Bifidobacterium	+1.32 ± 0.54	+0.13 ± 0.48	0.004
Faecalibacterium prausnitzii	+0.87 ± 0.61	+0.11 ± 0.39	0.010
Roseburia	+0.62 ± 0.57	+0.12 ± 0.51	0.026
Escherichia coli	–0.75 ± 0.49	–0.09 ± 0.32	0.017

In the group treated with iSGLT2, we observed a significant increase in the abundance of beneficial species, especially Bifidobacterium (mean Δ: +1.32 ± 0.54, $p < 0.01$) and Faecalibacterium prausnitzii (Δ: +0.87 ± 0.61, p = 0.042). In parallel, Escherichia coli

recorded a decrease (Δ: –0.75 ± 0.49, p = 0.036), suggesting a positive effect on bacterial balance. In the IDPP4 group, changes were minimal and not statistically significant. Δ comparison between groups showed apparent differences in favour of empagliflozin for all four species.

The hypothesis is confirmed.

Hypothesis 6 Hypothesis 5: Changes in the microbiota correlate with improvements in metabolic and inflammatory parameters, suggesting a mechanistic role of the microbiota in the benefits of iSGLT2 treatment

The results highlighted significant correlations between the relative growth of beneficial species and improvements in metabolic and inflammatory status. For example, an increase in the abundance of the genus *Bifidobacterium* was associated with a significant decrease in HbA1c (Spearman coefficient = –0.41, p = 0.032) and IL-6 (coef = –0.38, *p* = 0.047). *Faecalibacterium prausnitzii was* also negatively correlated with hs-CRP and positively correlated with HDL-cholesterol. In multivariate regression, *Bifidobacterium* remained an independent predictor of HbA1c reduction, with a coefficient β = –0.27, 95% CI: –0.49 –0.06, *p* = 0.014.

ANALYSIS OF RESULTS. CONCLUSIONS

Both therapeutic classes improve metabolic parameters, but with differences in magnitude and mechanisms, favoring SGLT2 inhibitors. Treatment with iSGLT2 alone has a significant influence on the intestinal microbiota. The interdependence between metabolism, inflammation and microbiota is more evident in the case of treatment with iSGLT2, which is proven by the multiple correlations between these components. Based on clinical and biological response, patients treated with iSGLT2 were classified into four clinical-biological patterns: complete, partial, non-response, and paradoxical. This stratification was not possible in the DPP-4i group, where the variability of the response was lower and the biological changes were more homogeneous, but also more limited in magnitude. This suggests that iSGLT2, through its multiple influences, can generate differentiated response profiles, providing opportunities for precision medicine. Both classes have favorable safety profiles, but iSGLT2 brings pleiotropic benefits. SGLT2 inhibitors consistently influence the low-intensity inflammatory background associated with DM type 2 2, supporting its use in profiles with high inflammatory risk (e.g. metabolic syndrome, intestinal dysbiosis, obesity).

FUTURE DIRECTIONS OF CLINICAL RESEARCH AND DEVELOPMENT

The practical applicability of this doctoral thesis includes the selection of the therapeutic class according to the patient's profile, the integration of inflammatory markers in the current assessment, the analysis of the intestinal microbiota as a clinical tool, the guidance of treatment based on response patterns, the reconsideration of the role of nutrition and adjuvant therapy with pre and probiotics.

This study can serve as a starting point for future research and new directions. First, MI analyses can be **extended** and correlated with other modern therapeutic classes used in the treatment of type 2 DM, such as GLP-1 agonists or dual agonists. Changes in MI during treatment can be used as a **marker of therapeutic response**. This approach would enable dynamic therapeutic interventions by adjusting the drug class or adding adjuvants (probiotics, postbiotics) based on the documented microbiotic response.

In addition to its therapeutic role, the gut microbiome can become a valuable screening **and prevention** tool. Early identification of patients with a dysbiotic profile – even before the onset of overt hyperglycemia – could allow early interventions through diet, lifestyle or supplementation with prebiotics/probiotics, thus helping to prevent progression to type 2 DM. The results presented in this paper highlight the importance of integrating clinical, biological, and microbial data in developing personalized therapeutic strategies, which extend beyond the traditional paradigm based solely on glycemic values. The correlations of metabolic, inflammatory, and microbial parameters can serve as starting points for multidisciplinary research, allowing for even more precise differentiation of response patterns. Last but not least, **digitization and data integration**, the use of artificial intelligence algorithms, and multi-year monitoring, which simultaneously track metabolic, inflammatory, and microbiotic parameters, will provide a comprehensive picture of the patient with DM2. This dynamic and personalized approach enables early adaptation of therapy, leading to optimal metabolic control and the prevention of complications. In the future, integrating this data into an intelligent digital system could radically transform the way diabetes is managed, from prevention to maintenance treatment.