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CIÊNCIAS VETERINÁRIAS

The influence of Renin-Angiotensin System (RAS) in Pets Diabetic Dysmotility

Marisa Manuela Esteves Monteiro

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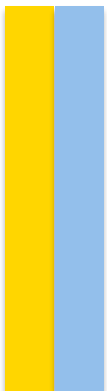

INSTITUTO DE **CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**

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The influence of Renin-Angiotensin System (RAS) in Pets Diabetic Dysmotility

Thesis submitted for a Doctoral Degree in Veterinary Sciences;
Doctoral Programme of University of Porto (School of Medicine and Biomedical Sciences)

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Marisa Manuela Esteves Monteiro

A influência do Sistema Renina Angiotensina (SRA) na dismotilidade diabética em animais de companhia

Tese de Candidatura ao grau de Doutor em Ciências Veterinárias;
Programa Doutoral da Universidade do Porto
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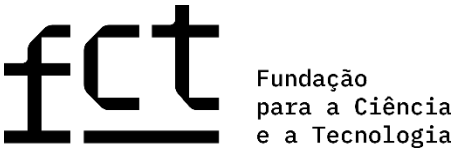

Partes desta investigação foram realizadas em:

- Laboratório de Farmacologia, Departamento de Ciências do Medicamento da Faculdade de Farmácia da Universidade do Porto
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“Na noite em que tu nasceste,
a Lua sorriu com tanto contentamento
que até as estrelas curiosas,
se puseram à espreita para te ver,
e a brisa da noite sussurrou:
“A vida nunca mais será igual.”

Na noite em tu nasceste,
trouxeste alegria e magia ao mundo,
porque nunca existiu ninguém como tu…
No mundo inteiro!”

Vi, esta tese é para ti.
Tudo é por ti.

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List of scientific papers

1. **Histomorphometry Changes and Decreased Reactivity to Angiotensin II in the Ileum and Colon of Streptozotocin-Induced Diabetic Rats.**
   Marisa Esteves-Monteiro, Daniela Menezes-Pinto, Mariana Ferreira-Duarte, Patrícia Dias-Pereira, Manuela Morato, Margarida Duarte-Araújo. Int J Mol Sci. 2022 Oct 31;23(21):13233. doi: 10.3390/ijms232113233. PMID: 36362021; PMCID: PMC9656372.

2. **Refinement of an effective streptozotocin-induced diabetic model in rats: fasting and analgesia.**
   Marisa Esteves-Monteiro, Daniela Menezes-Pinto, Mariana Ferreira-Duarte, Manuela Morato, Margarida Duarte-Araújo. Manuscript submitted to Journal of Applied Animal Welfare Science (Submission ID: 239065425) and under revision.

3. **Oxidative Stress and Histomorphometric Remodeling: Two Key Intestinal Features of Type 2 Diabetes in Goto-Kakizaki Rats.**
   Marisa Esteves-Monteiro, Mariana Ferreira-Duarte, Claúdia Vitorino-Oliveira, José Costa-Pires, Sara Oliveira, Paulo Matafome, Manuela Morato, Patrícia Dias-Pereira, Vera Marisa Costa, Margarida Duarte-Araújo. Int J Mol Sci. 2024 Nov 12;25(22):12115. doi: 10.3390/ijms252212115. PMID: 39596183; PMCID: PMC11594829. Corresponding author.

4. **Differential effects of Losartan and Finerenone on diabetic remodeling, oxidative stress and ACE activity in the gastrointestinal tract of streptozotocin-induced diabetic rats**
   Marisa Esteves-Monteiro, Cláudia Vitorino-Oliveira, Joana Castanheira-Moreira, Mariana Ferreira-Duarte, Patrícia Dias-Pereira, Vera Marisa Costa, Manuela Morato & Margarida Duarte-Araújo. Manuscript in preparation.

5. **Ultrasonographic evaluation of the normal gastrointestinal wall in dogs and cats: a systematic review on study design and imaging outcomes**
   Marisa Esteves-Monteiro, Margarida Duarte-Araújo, Clara Landolt, Cláudia Sofia Baptista. Manuscript in preparation.

6. **Exploring Gastrointestinal Health in Diabetic Cats: Insights from Owner Surveys, Ultrasound, and Histopathological Analysis**
   Marisa Esteves-Monteiro, Diogo Coutinho, Clara Landolt, Patrícia Dias-Pereira, Cláudia Sofia Baptista, Margarida Duarte-Araújo. Manuscript ready for submission.

Patent:

Margarida Duarte-Araújo, Marisa Esteves- Monteiro, Manuela Morato.
Pedido provisório de Patente: PT 119319; Data do pedido: 14/03/2024
Pedido de Patente Internacional nº PCT/IB2025/052714; Data do pedido: 14/03/2025
Repurposing the use of angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists to prevent and/or treat gastrointestinal complications associated with Diabetes mellitus

List of scientific papers coauthor

1. **ACE and ACE2 catalytic activity in the fecal content along the gut**
   Mariana Ferreira-Duarte, Lilian Caroline Gonçalves Oliveira, Clara Quintas, Marisa Esteves-Monteiro, Margarida Duarte-Araújo, Tersa Sousa, Dulce Elena Casarini, Manuela Morato. Neurogastroenterol Motil. 2023 Sep;35(9):e14598. doi: 10.1111/nmo.14598. Epub 2023 Apr 13. PMID: 37052403..

2. **Interaction between the Renin–Angiotensin System and Enteric Neurotransmission Contributes to Colonic Dysmotility in the TNBS-Induced Model of Colitis**
   Mariana Ferreira-Duarte, Tiago Rodrigues-Pinto, Teresa Sousa, Miguel A. Faria, Maria Sofia Rocha, Daniela Menezes-Pinto, Marisa Esteves-Monteiro, Fernando Magro, Patrícia Dias-Pereira, Margarida Duarte-Araújo, Manuela Morato. Int J Mol Sci. 2021 May 3;22(9):4836. doi: 10.3390/ijms22094836. PMID: 34063607; PMCID: PMC8125095.

3. **2,4,6-trinitrobenzenesulfonicacid-induced colitis in *Rattus norgevicus*: a categorization proposal**
   Mariana Ferreira-Duarte, Tiago Rodrigues-Pinto, Daniela Menezes-Pinto, Marisa Esteves-Monteiro, Salomé Gonçalves-Monteiro, Sara Capas-Peneda, Fernando Magro, Patrícia Dias-Pereira, Manuela Morato, Margarida Duarte-Araújo. Exp Anim. 2021 May 13;70(2):245-256. doi: 10.1538/expanim.20-0113. Epub 2021 Feb 3. PMID: 33536378; PMCID: PMC8150238.

Although none of these papers are directly included in this thesis, they were instrumental in the acquisition of knowledge regarding the manipulation of laboratory animals, the development of functional experimental protocols, the renin-angiotensin-aldosterone system, and the exploration of the activity of angiotensin-converting enzyme in the gastrointestinal tract.

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List of Abbreviations

α-SMA	α-smooth muscle actin
ACE	Angiotensin Converting Enzyme
ACE2	Angiotensin Converting Enzyme 2
ACEi	Angiotensin Converting Enzyme inhibitors
AsSDKP	N-acetyl-seryl-aspartyl-lysyl-proline
ACh	Acetylcholine
Ang	Angiotensin
AGEs	Advanced Glycation Endproducts
ARBs	Angiotensin Receptor Blockers
AT1R	Angiotensin Receptor type 1
AT2R	Angiotensin Receptor type 2
CM	Circular muscle
DALYs	Disability-Adjusted Life Years
DAN	Diabetic Autonomic Neuropathy
DC	Distal Colon
DGAV	Direção-Geral de Alimentação e Veterinária
DRIs	Direct renin inhibitors
DM	*Diabetes mellitus**
ECM	Extracellular matrix
ENS	Enteric Nervous System
FIV	Feline Immunodeficiency Virus
GI	Gastrointestinal
GIT	Gastrointestinal tract
GK	Goto-Kakizaki
GSH	Glutathione
GSSG	Oxidized glutathione
H&E	Hematoxylin and Eosin
h-HL	Hip-His-Leu
ICC	Interstitial Cells of Cajal
ITT	Insulin Tolerance Test
LM	Longitudinal muscle
MC	Middle Colon
MMPs	Matrix Metalloproteinases
MRAs	Mineralocorticoid Receptor Antagonists
NO	Nitric Oxide

ORBEA	Organismo Responsável pelo Bem-Estar Animal
PAS	Periodic Acid–Schiff
PC	Proximal Colon
PO	*Per os*
PMSF	Phenylmethylsulfonyl fluoride
RAGE	Advanced Glycation Endproducts Receptor
RAAS	Renin Angiotensin Aldosterone System
ROS	Reactive Oxygen Species
RV	Reference Value
SMCs	Smooth Muscle Cells
STZ	Streptozotocin
tGSH	Total Glutathione
T1D	Type 1 Diabetes*
T1DM	Type 1 Diabetes*
T2D	Type 2 *Diabetes mellitus**
T2DM	Type 2 *Diabetes mellitus**
TGF-β1	Transforming growth factor-beta 1
Z-FHL	Benzyloxycarbonyl-phenylalanyl-histidyl-leucine

* **Note on Terminology:**

Throughout this thesis, the terms **"diabetes mellitus" (DM), "type 1 diabetes mellitus" (T1DM), and "type 2 diabetes mellitus" (T2DM)** appear in sections that were previously ready, published or submitted. However, in more recent work, the simplified terms **"diabetes" (D), "type 1 diabetes" (T1D), and "type 2 diabetes" (T2D)** are used, reflecting the evolving preference in scientific literature and guidelines, including those from the International Diabetes Federation.

Abstract

Diabetes is a complex, chronic, and progressive metabolic disorder, currently incurable, that can affect nearly every organ system. Its prevalence is alarmingly high worldwide, affecting individuals across all age groups, and it is also one of the most common endocrinopathies in cats and dogs. Gastrointestinal complications are common and clinically significant, often associated with substantial morbidity, affecting up to 75% of diabetic patients. Despite this high prevalence, there is a significant lack of knowledge and treatment options for these complications. Additionally, little is known about gastrointestinal alterations in diabetic cats and dogs. The renin-angiotensin-aldosterone system (RAAS) has been extensively studied in the context of cardiovascular and renal health, where it is known to regulate vascular tone and electrolyte homeostasis. Moreover, there is growing evidence of a relationship between exacerbation of RAAS activation and the development of diabetes-related complications. The primary aims of this thesis were to characterize diabetic gut changes in pets and animal models of both Type 1 (T1D) and Type 2 diabetes (T2D), and to explore the pathophysiological role of the RAAS in these changes, with a focus on evaluating the potential preventive effects of angiotensin II (Ang II) type I receptor (AT_1R) antagonists (ARBs), like losartan.

The streptozotocin (STZ)-induced model was used to study T1D, while Goto-Kakizaki rats were employed to study T2D. Both models exhibited hyperglycemia compared to controls and displayed typical diabetic signs, including polyphagia and polydipsia. STZ-induced rats experienced significant weight loss, whereas GK rats and controls showed similar weight gains. Both models demonstrated remodeling of the gastrointestinal wall, characterized by increased thickness across various segments of the gut, along with a decreased local GSH/GSSG ratio, which indicates oxidative stress. In STZ animals, we also observed a decreased functional response to Ang II. These animals exhibited increased systemic activity of angiotensin converting enzyme (ACE) and ACE2; however, locally in the gastrointestinal tract, only ACE levels were elevated, suggesting a shift in the RAAS towards enhanced Ang II production. In the losartan-treated STZ group, the thickening of muscular layers throughout the gastrointestinal tract was prevented, resembling control animals. Additionally, the tGSH and GSH/GSSG ratio were normalized, reinforcing the potential of losartan as a preventive measure for gastrointestinal complications in diabetes.

In diabetic cats with no prior history of gastrointestinal disease, more than 80% exhibited gastrointestinal signs, including vomiting and diarrhea. Additionally, nearly half of the owners reported changes in their cats' defecation behavior, particularly defecating outside the litter box. Ultrasound evaluations revealed increased wall thickness in the stomach,

duodenum, and jejunum, while the ileum and colon had normal thickness. Histopathological analysis confirmed the increased thickened muscular layers throughout the gastrointestinal tract, accompanied by inflammatory infiltrates and collagen deposits. Preliminary results in diabetic dogs indicate that, unlike what was observed in cats, only half of the animals exhibited gastrointestinal manifestations of diabetes, shortly after diagnosis. However, ultrasonographic evaluation of the gastrointestinal tract suggests a potential thickening of the stomach, duodenum, and jejunum, while the ileum and colon consistently appeared within normal limits. Further investigation is required to confirm and support these findings.
Taken together, the findings of this thesis are highly innovative. We demonstrate that both T1D and T2D models exhibit significant histomorphometric changes in the gut wall, characterized by increased thickness. For the first time, we identify signs of locally increased oxidative stress and a shift in the local RAAS, as evidenced by increased ACE activity. Furthermore, diabetic cats appear to experience gastrointestinal clinical signs and intestinal remodeling similar to those observed in human patients and experimental diabetes models.
From a translational standpoint, the present data suggest that ARBs, such as losartan, may help prevent diabetic gastrointestinal complications, enhancing the quality of life for both pets and human patients.

Resumo

A diabetes é uma doença metabólica crónica, complexa e progressiva, atualmente incurável, que pode afectar praticamente todos os sistemas orgânicos. A sua prevalência é alarmantemente elevada a nível global, atingindo indivíduos de todas as faixas etárias, sendo também uma das endocrinopatias mais comuns em gatos e cães. As complicações gastrointestinais são frequentes e clinicamente significativas, frequentemente associadas a uma morbilidade substancial, afectando até 75% dos doentes diabéticos. Apesar desta elevada prevalência, existe um conhecimento limitado sobre estas complicações, bem como opções terapêuticas escassas. Adicionalmente, as alterações gastrointestinais em gatos e cães diabéticos permanecem muito pouco estudadas.

O sistema renina-angiotensina-aldosterona (SRAA) tem sido amplamente investigado no contexto da saúde cardiovascular e renal, sendo conhecido pelo seu papel na regulação do tónus vascular e da homeostasia electrolítica. Além disso, há um crescente corpo de evidência que sugere uma relação entre a exacerbação da ativação do SRAA e o desenvolvimento de complicações associadas à diabetes. Os principais objetivos desta tese foram caracterizar as alterações gastrointestinais associadas à diabetes em animais de companhia e em modelos animais de diabetes tipo 1 (DT1) e tipo 2 (DT2), bem como explorar o papel fisiopatológico do SRAA nestas alterações, com especial enfoque na avaliação dos potenciais efeitos preventivos dos antagonistas dos recetores do tipo 1 da angiotensina II (AT_1R), como o losartan.

Para o estudo da DT1 foi utilizado o modelo induzido por estreptozotocina (STZ), enquanto o modelo de ratos Goto-Kakizaki foi empregue para o estudo da DT2. Ambos os modelos apresentaram hiperglicemia em comparação com os controlos e exibiram sintomas típicos de diabetes, incluindo polifagia e polidipsia. Os ratos induzidos por STZ sofreram uma perda de peso significativa, enquanto os ratos GK e os controlos apresentaram aumentos de peso semelhantes. Ambos os modelos demonstraram remodelação da parede gastrointestinal, caracterizada por um aumento da espessura em vários segmentos do tracto digestivo, acompanhado por uma diminuição da razão GSH/GSSG local, indicando a presença de stress oxidativo. Nos animais STZ, observou-se ainda uma resposta funcional diminuída à angiotensina II. Estes animais apresentaram um aumento da atividade sistémica da enzima de conversão da angiotensina (ECA) e da ECA2; contudo, no tracto gastrointestinal apenas os níveis de ECA estavam elevados, sugerindo um desvio local do SRAA para uma produção aumentada de angiotensina II. No grupo de ratos STZ tratados com losartan, a hipertrofia das camadas musculares ao longo do tracto gastrointestinal foi prevenida, assemelhando-se aos animais controlo. Adicionalmente, os

níveis de tGSH e a razão GSH/GSSG foram normalizados, reforçando o potencial do losartan como estratégia preventiva para as complicações gastrointestinais da diabetes.

Em gatos diabéticos sem historial prévio de doença gastrointestinal, mais de 80% apresentaram sintomas gastrointestinais, incluindo vómitos e diarreia. Além disso, cerca de metade dos tutores relatou alterações no comportamento de defecação dos seus gatos, particularmente defecação fora da caixa de areia. As avaliações ecográficas revelaram um aumento da espessura da parede gástrica, do duodeno e do jejuno, enquanto o íleo e o cólon mantiveram espessuras normais. A análise histopatológica confirmou a hipertrofia das camadas musculares ao longo do trato gastrointestinal, acompanhada por infiltrados inflamatórios e depósitos de colagénio. Resultados preliminares em cães diabéticos indicam que, ao contrário do observado nos gatos, apenas metade dos animais apresentou manifestações gastrointestinais da diabetes aquando ou após o diagnóstico de diabetes. No entanto, a avaliação ecográfica do tracto gastrointestinal sugere um potencial espessamento do estômago, duodeno e jejuno, enquanto o íleo e o cólon aparentaram manter-se dentro dos limites normais. Investigações adicionais são necessárias para confirmar e aprofundar estes achados.

Em suma, os resultados desta tese são altamente inovadores. Demonstramos que os modelos de DT1 e DT2 apresentam alterações histomorfométricas significativas na parede intestinal, caracterizadas por um aumento da espessura. Pela primeira vez, identificámos sinais de um aumento localizado do stress oxidativo e um desvio do SRAA, evidenciado pelo aumento da atividade da ECA. Além disso, os gatos diabéticos parecem apresentar sintomas gastrointestinais e remodelação intestinal semelhantes aos observados em doentes humanos e em modelos experimentais de diabetes.

Do ponto de vista translacional, os dados obtidos sugerem que os antagonistas dos recetores AT_1R, como o losartan, poderão ajudar a prevenir complicações gastrointestinais associadas à diabetes, contribuindo para a melhoria da qualidade de vida tanto de animais de companhia como de doentes humanos.

Chapter 1: State of the art

1. Diabetes

Diabetes mellitus, or just diabetes, is a serious chronic disorder that has become one of the fastest-growing global health emergencies of the 21st century[1]. According to the International Diabetes Federation, approximately 537 million adults worldwide (20 to 79 years old) had diabetes in 2021 (around 10.5% of the world adult population). This number is projected to rise to around 643 million by 2030 and 783 million by 2045[2]. Portugal is no exception to the high incidence of diabetes, and actually has a higher prevalence compared to the global average. In 2021, approximately 1.1 million adult Portuguese individuals were affected by diabetes, with an estimated prevalence of 14.1%[3].

Diabetes is a significant global cause of mortality, contributing to 3.7 million adult deaths worldwide in 2012, including death by complications associated with the disease[1]. By 2021, this number had risen to approximately 6.7 million, accounting for 12.2% of all global deaths in the adult population[2]. Besides the significant mortality rate, the importance of diabetes as a public health problem is also related to considerable morbidity and economic impact. In 2019, diabetes was considered a leading cause of disability and was the eighth most common global risk factor for disability-adjusted life years (DALYs), accounting for 79.2 million DALYs, representing an increase of 25% between 1990 and 2021[4]. Regarding the economic ramifications of diabetes, they extend beyond direct expenses like medical bills, medication, and diagnostic tests. Indirect costs, including loss of wages and productivity, as well as intangible expenses such as diminished functionality, heightened pain, and reduced quality of life, also significantly contribute to its overall impact[5]. The global economic burden amounted to approximately U.S. $1.3 trillion in 2015 and is projected to escalate to $2.1 trillion by 2030[6].

1.1. Classification

Diabetes is a metabolic disorder characterized by dysregulation of carbohydrate metabolism caused by insulin deficiency, defect in insulin action, or both. This dysregulation manifests as both underutilization of glucose as an energy source and overproduction through inappropriate gluconeogenesis and glycogenolysis, leading to hyperglycemia[7]. Individuals with diabetes typically exhibit random plasma glucose levels exceeding 200 mg/dL, irrespective of the time elapsed since their last meal[8]. This disorder is traditionally categorized into various clinical types, including type 1 and type 2 diabetes, gestational diabetes, and other specific types arising from distinct causes such as genetic predispositions, exocrine pancreatic disorders, and the use of medication like

glucocorticoids. While diabetes includes these various forms, the two main types are type 1 diabetes (T1D) and type 2 diabetes (T2D) (figure 1)[8].

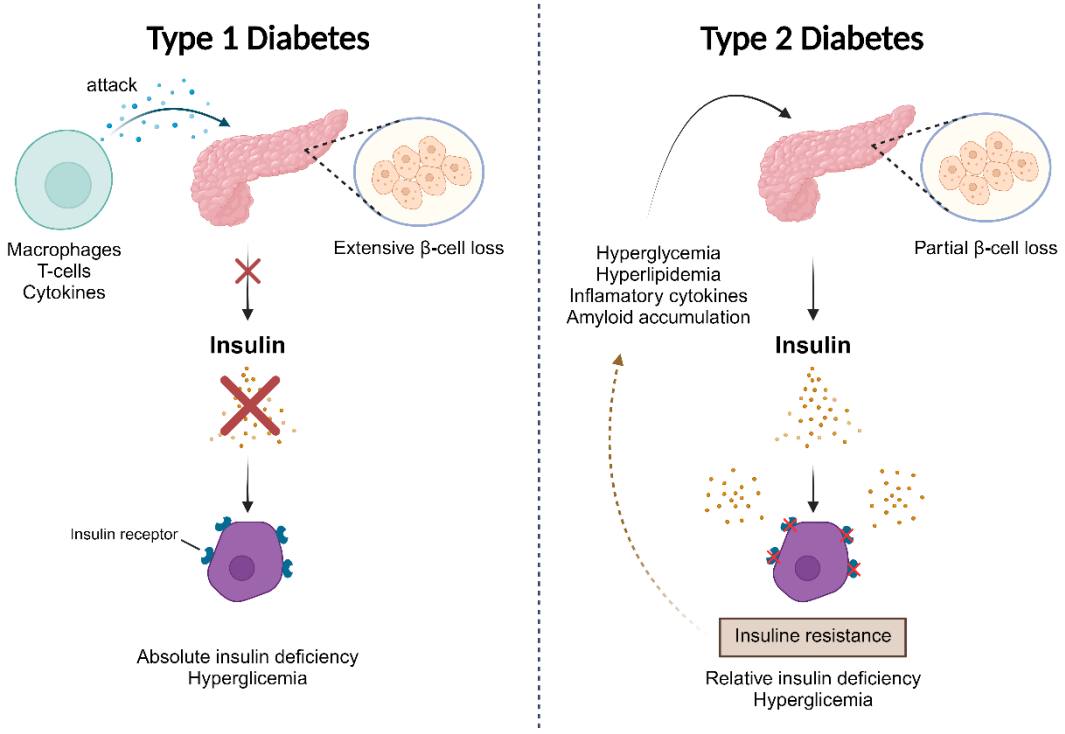

Figure 1 - Pathogenesis of type 1 diabetes (T1D) and type 2 diabetes (T2D). In T1D, the destruction of pancreatic β-cells leads to an absolute deficiency of insulin, causing hyperglycemia. Conversely, in T2D, pancreatic β-cells are damaged by factors such as hyperglycemia, hyperlipidemia, cytokines, and amyloids. Although these cells still produce insulin, the amount is insufficient to overcome insulin resistance, resulting in a relative insulin deficiency and subsequent hyperglycemia.

T1D is a chronic autoimmune disease and comprises 5-10% of all cases of diabetes, but the prevalence has been increasing[9,10]. Most cases of T1D occur in children under the age of 15, with an increasing prevalence observed in children younger than 5 years old[10]. Under normal conditions, pancreatic β-cells release insulin in response to elevated blood glucose levels. However, in this type of diabetes, there is a complete absence of insulin secretion[11]. This happens because of a cellular-mediated autoimmune inflammatory reaction targeting pancreatic β-cells, leading to a complete deficiency in insulin production. Since the pancreas cannot produce insulin, individuals with T1D require daily insulin administration for management, which is why this condition is also referred to as Insulin-Dependent Diabetes[9]. The causes of pancreatic β-cell destruction remain a topic of debate, with ongoing research and various theories attempting to uncover the exact triggers of T1D [12,13]. However, the incidence rate appears to depend on genetic susceptibility, as specific

combinations of alleles and genes are significantly associated with an increased risk of developing T1D [14]. Markers of immune-mediated destruction of β-cells include several islet cell autoantibodies (GAD65, IA-2, and ZnT8) as well as autoantibodies to insulin and the antibody profile is a strong predictor of the rate at which diabetes progresses[15]. Additionally, environmental factors such as viral infections (including rubella, rotavirus, and cytomegalovirus), toxins, dietary factors during infancy, and other variables also seem to play a role[14,16].

T2D is characterized by a combination of insulin resistance in insulin-sensitive tissues and a relative deficiency of insulin caused by dysfunctional pancreatic β-cells, making up 90 to 95% of all diabetes cases[9,17]. Individuals at risk of developing T2D typically exhibit an initial stage of insulin resistance, which is compensated by increased insulin production by the pancreatic β-cells. As time progresses, this compensatory mechanism becomes insufficient to meet the body's insulin demands. In the first phase, individuals may maintain normal glucose levels through increased insulin secretion, despite the underlying insulin resistance. With continued progression, insulin sensitivity further diminishes and β-cell mass decreases, leading to non-compensated hyperglycemia[9,18]. T2D emerges from a multifaceted interplay of genetic predisposition, metabolic conditions, and environmental influences[19]. Among these factors, obesity stands out as a significant risk factor, exerting a profound impact on T2D prevalence. Physical inactivity coupled with the consumption of high-energy diets are recognized as primary drivers behind the escalating rates of T2D[20]. Additionally, smoking, excessive alcohol intake, advancing age, familial history of T2D, gestational diabetes, certain medications, as well as stress and depression, contribute to the intricate web of risk factors[21]. Nonetheless, approximately 20% of T2D patients in Europe are non-obese, with an even higher proportion observed in Asia. In these cases, the development of hyperglycemia is primarily attributed to a complex interplay between impaired insulin secretion and insulin resistance. This suggests that both factors, rather than obesity, are crucial in the pathogenesis of T2D in non-obese individuals[22].

1.2. Symptoms and complications

The most common symptoms of diabetes are often referred to as the "4 P's" of diabetes: polyuria, polydipsia, polyphagia, and weight loss ("perda de peso" in Portuguese)[23].

Whether in T1D or T2D, ineffective insulin leads to hyperglycemia because, without adequate insulin the movement of glucose from plasma into body cells is impaired[24]. Under normal conditions, glucose is filtered by the glomerulus and reabsorbed entirely by the proximal tubule through Na^+-coupled apical uptake via SGLT1/2 glucose carriers and basolateral facilitated diffusion via GLUT1/2 glucose transporters. As plasma glucose levels

increase, the concentration of glucose in the glomerular filtrate also rises. When this concentration surpasses the resorptive capacity of the proximal tubule (the renal threshold), glucose is excreted with urine (glucosuria). The presence of glucose in urine acts as an osmotic agent, leading to an increased volume of urine excretion – osmotic diuresis[25]. Due to the increased water loss through urine, individuals with diabetes need to drink more water to compensate for this fluid loss[24], thus explaining both polyuria and polydipsia.

Blood glucose levels are determined by the dynamic balance between glucose consumption and production. Glucose production primarily arises from gluconeogenesis and hepatic glycogen breakdown, while glucose consumption depends on peripheral tissue utilization[26]. The liver plays a central role in maintaining glucose metabolism by performing opposite functions during hyperglycemic (glucose uptake and glycogen synthesis) and hypoglycemic states (glycogenolysis and gluconeogenesis) and is crucial in the pathogenesis of diabetes[26,27]. In individuals with diabetes, despite the existing hyperglycemia, the lack of glucose uptake by cells triggers the liver to initiate glycogenolysis, followed by gluconeogenesis, further elevating glycemia. Since glucose still cannot enter the cells, processes like lipolysis in adipose tissue and proteolysis in muscle tissue start to occur. These processes contribute to reduced body mass and weight loss[28]. The glycerol from lipolysis is used as a substrate for gluconeogenesis[29], while triglycerides are converted into ketone bodies in the liver[30]. The persistent lack of intracellular glucose creates a state of cellular starvation, leading to polyphagia as a compensatory response. However, this increased food intake does not result in weight gain; instead, it exacerbates hyperglycemia, along with associated polyuria and dehydration[31].

Over time, persistent hyperglycemia can profoundly impact nearly every organ system in the body, impairing their function and potentially precipitating organ failure[32]. A classical categorization of diabetes complications include macrovascular complications, microvascular complications, and neuropathy[33]. The mechanism underlying vascular and nerve damage stems from their permeability to glucose even in the absence of insulin. This allows glucose levels within the vascular endothelium and nerve tissue to mirror those in the plasma[31].

Macrovascular complications are majorly due to the atherosclerotic narrowing of large arteries and veins, leading to cardiovascular, cerebrovascular or peripheral artery diseases. Cardiovascular disease is the principal cause of death in most of the diabetic population[33]. Diabetes-induced cardiomyopathy is characterized by significant cardiac fibrosis, intensified by the overproduction of oxidative free radicals. These radicals disrupt myocardial cells, leading to the dysregulation of cellular calcium homeostasis, contractile dysfunction, myocardial remodeling, and, ultimately, cardiomyocyte death. Moreover, oxidative stress surpasses the antioxidant protective system in diabetic patients, exacerbating the

condition[32]. Cerebrovascular diseases, such as stroke and ischemia, arise from the atherosclerotic narrowing of intracranial vessels and the carotid artery. Meanwhile, peripheral artery disease denotes the atherosclerotic occlusive disorder affecting the lower extremities, frequently entailing a heightened risk of extremity amputation[33].

Conversely, diabetic microvascular complications primarily stem from impaired vascular permeability, which impacts various tissues and organs throughout the body. This includes the kidneys, leading to diabetic nephropathy, and the retina, resulting in diabetic retinopathy[32]. Diabetic retinopathy stands as the leading cause of blindness in the adult population (individuals aged 20 to 74 years)[34]. Diabetic nephropathy denotes the pathological dysfunction of the kidneys in diabetic patients and is recognized as the primary initiator of end-stage renal failure[33,34].

Neuropathic complications in diabetes stem from various mechanisms, including neurovascular deficiency, metabolic insult due to hyperglycemia, autoimmune damage, and heightened oxidative stress. These factors can contribute to diabetic autonomic neuropathy (DAN), a condition that impacts numerous organ systems, such as cardiovascular, genitourinary, and gastrointestinal functions[35].

Additional chronic complications of diabetes encompass skin disorders, periodontal disease, and bone disease characterized by diminished bone mineral density, resulting in an increased risk of bone fractures[32].

1.3. Gastrointestinal complications of Diabetes

Gastrointestinal (GI) complications of diabetes are highly prevalent and constitute a significant cause of morbidity, affecting up to 75% of diabetic patients, which influences their health status and quality of life[36,37]. A study investigating the impact of GI complications in diabetic patients found that these complications significantly decrease health-related quality of life. This aspect of health encompasses physical, emotional, and social functions. The study revealed that GI symptoms affect not only physical functioning and general health perceptions but also vitality, social functioning, and emotional and mental health[38]. However, awareness of these complications among physicians is often limited, with scant knowledge and treatment options available[39,40]. Despite these alterations being described since 1971[41], the precise mechanism underlying diabetes-related GI complications remain far less understood compared to diabetic retinopathy or nephropathy[42]. Consequently, many patients remain undiagnosed and untreated, as the GI tract has not traditionally been associated with diabetes[43]. Even in children with T1D, GI complaints are very common, and the diabetic complained of more intense symptoms than their peers without diabetes[44].

Currently, there is no cure for diabetic gastroenteropathy, and considerable controversy surrounds the mechanisms responsible for GI symptoms in diabetes[39,45]. Therefore, available treatments only aim to slow disease progression, alleviate symptoms, and manage complications[45].

The entire GIT appears to be affected by diabetes, with alterations observed from the esophagus to the rectum[40,46]. The classic GI symptoms of diabetes include post-prandial fullness with nausea, bloating, abdominal pain, diarrhea, and/or constipation (figure 2)[47].

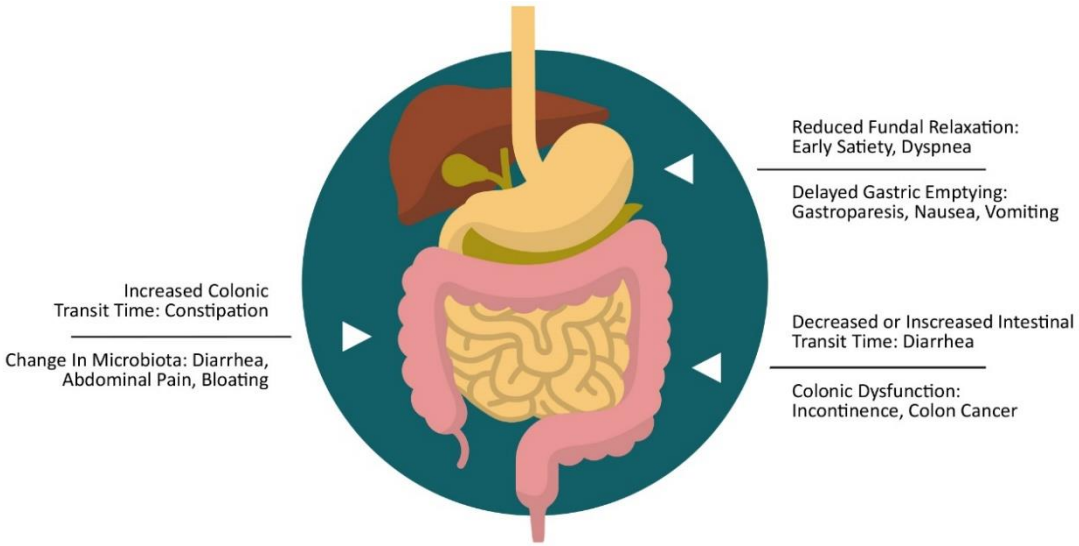

Figure 2 - Common gastrointestinal complications of diabetes.

1.3.1. Upper GI tract complications

The esophageal symptoms of diabetes arise from alterations in morphological and biomechanical properties, including increased stiffness, reduced compliance, and decreased sensitivity to distension, ultimately resulting in dysmotility[48,49]. Abnormalities observed in esophageal motility among diabetic patients include reduced lower esophageal sphincter tone and abnormal peristalsis characterized by diminished amplitude of esophageal contractions, reduced coordination, and prolonged esophageal transit, affecting up to 63% of patients[50]. DAN appears to drive these esophageal motility disorders. Additionally, gastroesophageal reflux disease, impacting as many as 41% of diabetic patients, further compounds the condition. Symptoms of esophageal dysmotility may manifest as heartburn after eating or drinking, chest pain, and dysphagia[46,51].

Gastroparesis is characterized by delayed gastric emptying in the absence of mechanical obstruction. It is considered one of the most recognized GI manifestations of diabetes and seems to affect up to 65% of patients with T1D and in up to 30% of patients with T2D[47]. Gastroparesis seems to be more common in women and may cause symptoms such as early satiety, anorexia, postprandial nausea, vomiting, bloating, postprandial fullness and upper abdominal pain[51,52].

Due to the delayed emptying of indigestible solids, bezoars are commonly found in diabetic gastroparesis. Postprandial antral contractions are often reduced in both frequency and amplitude. Additionally, diabetic patients may suffer from pyloric dysfunction or spasms, which can explain recurrent nausea and vomiting[51]. The combination of food retention in the stomach alongside accelerated gastric emptying creates a disparity between insulin activity and nutrient absorption[40]. This imbalance leads to inadequate postprandial glycemic control, resulting in fluctuating episodes of hyperglycemia and hypoglycemia[53].

The pathogenesis of diabetic gastroparesis is multifactorial and currently poorly understood[40]. Gastric emptying in healthy individuals is a sophisticated process that relies on the harmonious interaction between smooth muscle and autonomic nerves. This coordination occurs within the Interstitial Cells of Cajal (ICCs), often referred to as the pacemakers of the GI tract (GIT). ICCs play a pivotal role in integrating fundic tone, antral contractions, and pyloric relaxation to facilitate efficient postprandial emptying[46]. Elevated glycated hemoglobin level indicative of poor glycemic control is enough to cause disrupts in gastric coordination and emptying[40]. Also, the presence of DAN and macro and microvascular complications are accepted risk factors for the development of diabetic gastroparesis[40,54]. Indeed, a decrease in the number of myenteric neurons in the stomach has been reported, along with phenotypic changes in these neurons[54]. These alterations have been associated with unfavorable extracellular conditions such as hyperosmolarity, low nutrient availability, and oxidative stress[55]. Other factors that may have a role in pathogenesis includes impaired inhibitory nitric oxide containing nerves and smooth muscle fibrosis[40]. A study in gastric biopsies from diabetic patients showed a decrease in the expression of nitrergic neurons, especially in areas that also had reduced ICCs[56]. The loss of nitrergic neurons in diabetes has been linked to delayed gastric emptying, primarily due to the reduction of these neurons in the pylorus[57].

While gastroparesis itself does not directly elevate mortality rates, it is linked to a poorer prognosis compared to age- and gender-matched individuals with normal gastric emptying. Research indicates that individuals with gastroparesis have a 5-year survival rate of 67%, notably lower than the expected 81% survival rate in the general diabetic population[46].

1.3.2. Lower GI tract complications

Small intestinal and colorectal dysfunctions are prevalent in patients with longstanding diabetes, particularly those suffering from gastroparesis[40]. The pathogenesis of intestinal and colonic dysfunction in diabetes is multifactorial and may involve the accumulation of advanced glycation end-products (AGEs), damage to the enteric nervous system (ENS) or ICCs, and fibrosis of the muscular layers[58].

AGEs are naturally formed in the body, but their production increases under hyperglycemic conditions and disruptions to glucose-regulating pathways due to the increased availability of glucose. Moreover, the activation of the AGEs receptor (RAGE) also influences cellular functions, inducing oxidative stress and the generation of inflammatory factor[59]. The increased formation of AGEs and the RAGE activation have been recognized as pivotal factors in the progression of diabetes and its associated complications[60]. In studies involving rats with streptozotocin (STZ)-induced T1D, it was observed that both AGEs and RAGE were upregulated in the duodenum, jejunum, ileum[61], and colon[62]. The binding of AGEs in the nervous plexus appears to inhibit neuronal nitric oxide synthase (nNOS) and induce nerve damage, leading to deterioration of nerve structure and function[63]. The high concentration of AGE/RAGE observed in crypts, villi, and the brush border likely plays a role in the digestive and absorption disorders observed in affected animals. Furthermore, the accumulation of AGEs in smooth muscle and RAGE in neurons may contribute to motor disorders in the intestine and colon[61,64]. Additionally, there appears to be a correlation between AGE/RAGE accumulation and hypertrophy of the intestinal layers, mediated by the overexpression of connective tissue growth factor, vascular endothelial growth factor, and platelet-derived growth factor[62]. In rat ileum, a connection was established between altered contraction parameters, such as heightened sensitivity of intestinal contraction to distension stimulation and reduced contractibility, and the expression of AGE and RAGE[64]. Another explanation for the reduced contractibility is an impaired Ca^{2+} signaling in the smooth muscle cells of colon through increased activation of cAMP/PKA pathway caused by the association of AGEs and RAGE[65].

Several studies have indicated that DAN causes damage to ENS in both diabetic rats and humans with diabetes[55,66,67]. It has been reported a decrease in the number of myenteric neurons in duodenum[68], jejunum[55], ileum[69], cecum[70] and colon[67] along with phenotypic changes affecting homeostasis of the enteric plexus[55]. The reduction of neurons was directly implicated in motility alterations in diabetic patients due to disturbed signalling in the myenteric plexus. The reduction of neurons has been directly implicated in motility alterations in diabetic patients due to disrupted signalling in the myenteric plexus[55,71]. Besides DAN, there are other mechanisms proposed for the observed neuronal loss in the gut of diabetic patients, including increased apoptosis, oxidative stress and AGE/RAGE, and decreased levels of nerve growth factors[37,72].

Similarly to the stomach, the chemical coding of neurons in the diabetic gut is altered, leading to changes in neurotransmitter content[57]. This affects the balance between inhibitory and excitatory neurons, contributing to motility dysfunction. Inhibitory neurons are more severely impacted, particularly those producing neuronal nNOS, which generates nitric oxide (NO)[37]. NO is a key neurotransmitter that as an inhibitory mediating smooth muscle

relaxation in the GI tract, controls the blood circulation and may even present a neuroprotective function[73]. Loss of nitrergic expression in the diabetic GI tract has been documented in several species[32,66,73–75], and may occur due to two primary mechanisms: lack of nNOS expression due to a phenotypic switch or physical degeneration and loss of nitrergic neurons, often associated with AGE/RAGE accumulation[76] and increased apoptosis[77]. Enteric neurons exhibit responsiveness to glucose levels, suggesting a potential correlation between hyperglycemia and the activation of apoptotic pathways, which could account for the observed increase in apoptosis[56]. Furthermore, the production of reactive oxygen species (ROS) in the small intestine and colon have been shown to increase apoptosis of enteric neurons, particularly inhibitory neurons, exacerbating motility issues in diabetic patients[78].

Acetylcholine (ACh) is the most common neurotransmitter of the ENS, playing a crucial role in smooth muscle contraction and enteric motility[42,79]. However, the results concerning cholinergic innervation in diabetic guts are contradictory. Some studies report no significant alterations in cholinergic innervation or acetylcholinesterase activity in the diabetic gut[80], while other research indicates both an increase[42] and a deficit in cholinergic neurotransmission with an impaired response to exogenous ACh in ileum[81] and colon[75] of diabetic rats. This impairment is thought to be due to decreased ACh release or production and a reduction in acetylcholinesterase action, leading to a subsequent down-regulation of muscarinic receptors[81]. A more recent study concluded that cholinergic alterations are not solely responsible for the GI complications of diabetes. Other neurotransmitters and structural changes in the gut also play a significant role in these complications[82].

Diabetes has also been linked to reduced number of ICCs and injuries like swollen mitochondria and partial depletion of cells bodies or process in both diabetic humans and animal models of diabetes[66,83–86]. The damage and/or loss of ICC represents a very relevant contributing factor for diabetes related GI disorders as their impairment may cause increased excitability of the gut[86] by affecting the contractile pattern of smooth muscles, contributing to the observed dysrhythmia[75,84,].

Finally, mechanical factors may also contribute to intestinal and colonic disorders, since diabetes seems to cause increased thickness of the layers of the intestine and colon, leading to a stiffening of the intestinal wall and a reduction in its resting compliance and relaxation capacity, in proportion to the duration of diabetes[61,87,88]. This remodeling phenomenon is primarily attributed to an augmented synthesis of collagen type 1[89]. However, a comprehensive understanding of the diverse mechanisms underlying diabetic gut remodeling and the importance of these alterations in the diabetic dysmotility remain elusive.

The cumulative effects of diabetes-related alterations in the intestine and colon, as outlined earlier, manifest in symptoms such as chronic constipation, diarrhea, and incontinence (figure 3). These changes also exacerbate clinical conditions like colorectal cancer and inflammatory bowel disease, leading to poorer outcomes[40,45].

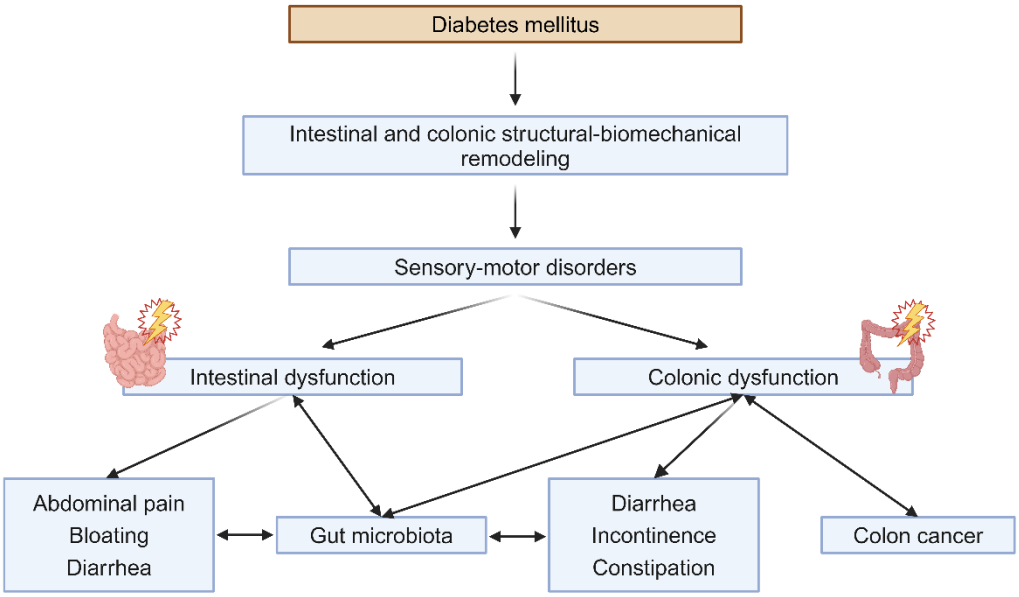

Figure 3 – Interaction between diabetes-induced intestinal and colonic changes and clinical consequences.

Constipation alternating with diarrhea is one of the most common symptoms[40,90]. Constipation can be explained by prolonged colonic transit time due to slower motility of the large bowel and affects up to 60% of diabetes patients[40]. Diarrhea affects up to 22% of patients with diabetes[40] and is typically intermittent, watery, painless, nocturnal, and may be associated with fecal incontinence in a third of the patients[91,92]. Episodes of incontinence are considered a troublesome symptom and may be attributed to the anal sphincter dysfunction, anorectal reflexes, and rectal motor-sensory dysfunction secondary to DAN, potentially exacerbated by acute hyperglycemic episodes[93]. Such episodes inhibit the sphincters and reduce rectal compliance[40,90]. Diabetic diarrhea can be intermittent, with episodes alternating between normal bowel movements and constipation[93].The mechanisms behind diarrhea in diabetics vary and are linked to neuropathy, disrupted intestinal peristalsis, and small intestinal bacterial overgrowth, which affects about 44% of diabetic patients and can cause abnormal bowel motility[93]. Even the changes in GI motility can influence the quantity, composition, and functionality of gut microbiota. This, in turn, may impact the integrity of the intestinal mucosa, the interaction with the ENS, and the performance of the smooth muscle layers[58].

There are reports indicating that individuals with diabetes, particularly T1D, have a higher predisposition to inflammatory bowel diseases like ulcerative colitis[93] and celiac disease[63,94]. Moreover, diabetes emerges as an independent risk factor for *Clostridium difficile* infection.

Additionally, a correlation has been suggested between diabetes and tumors of the GIT, more commonly colorectal malignancies, with diabetes being associated with poorer outcomes and responses to colorectal surgery[91]. Possible complications like megacolon, pseudo-obstruction, stercoral ulcer or perforation may (rarely) occur[40,95]

2. Diabetes in small animals

Diabetes one of the most common metabolic diseases in pets and the most common disorder of endocrine pancreas[96], occurring in 1 of every 300 patients (0.21% - 1.24% in cats[97] and 0.34% - 1.2% in dogs[98,99]). Since 2006 the incidence of diabetes in dogs has increased by and estimated 79.7%[100]. At least one of 100 dogs reaching 12 years of age develops diabetes[101] and the prevalence is even higher in genetically predisposed breeds like Samoyeds, Tibetan Terriers, Cairn Terriers, Miniature Schnauzers, Miniature Poodles, Pugs, Toy Poodles, and Australian Terriers[100,102]. Conversely German Shepherd Dogs, Golden retrievers and Boxers appear to have a reduced risk[103]. Breed appears to also influence the incidence of diabetes in cats, with Burmese cats being particularly predisposed. In Australia, approximately 1 in 50 Burmese cats is affected by diabetes[104]. Most cases of spontaneous diabetes occur in middle-aged dogs (7-12 years) and middle-aged to older cats (10-14 years)[96]. In dogs, female intact dogs are affected twice as often as males[103]. However, in cats, obese neutered males are more commonly affected than females[98].

Similar to human diabetes, this pathology in cats and dogs is associated with high levels of mortality and morbidity. In addition to the death related to complications of diabetes, a recent survey indicated that 10% of diabetic dogs are euthanized at the time of diagnosis, and another 10% within a year[105]. This is in line with what's described for cats since the mortality rate within the first 3-4 weeks after diabetes diagnosis is 11-17%[106] and the median survival time for diabetic cats is 516 days, with a range of 1 to 3468 days[107]. Diabetes in small animals also has a significant financial impact, with monthly insulin costs estimated at 70$ *per* dog, resulting in an annual cost of approximately 110$ million in the United States alone[100].

2.1. Canine diabetes

Canine diabetes is characterized by persistent hyperglycemia and insulin deficiency due to extensive β-cell loss[100], thus resembling human T1D. Although there is no evidence of T2D in dogs, several other forms of canine diabetes have been documented. These include diabetes that develops secondary to diestrus and pregnancy (gestational diabetes), early-

onset diabetes, which occurs in dogs less than a year old[102] and hyperadrenocorticism-associated diabetes[108]. The precise cause of pancreatic β cell dysfunction and destruction in dogs remains unclear. Nevertheless, exocrine pancreatic disease and immune-mediated mechanisms are suspected to be contributing factors[109]. In fact, a study showed that subclinical exocrine pancreatic disease was observed in 20% of diabetic dogs and other 20% showed evidence of pancreatitis with variable fibrosis[109]. Other studies showed that up to 33% of diabetic dogs present acute or chronic pancreatitis. Additionally, 36% of dogs with confirmed chronic pancreatitis had concurrent diabetes. So available evidence suggests that pancreatitis may serve as a causative or contributory element to diabetes in certain dogs. However, the broader prevalence and precise significance of exocrine inflammation in precipitating β-cell destruction remain uncertain[110].

Regarding the immune-mediated mechanisms causing diabetes, evidence suggests that autoantibodies are present in some diabetic dogs. One study found that anti-islet-cell antibodies were detected in 58% of diabetic dogs[111], anti-insulin antibodies were detected in 12.5% of untreated diabetic dogs[112] and proinsulin autoantibodies were found in 53% untreated diabetic dogs[113]. Histopathologicaly, pancreas of diabetic dog showed a significantly reduction in islet numbers and a substantial decrease in β-cells, while α- and δ-cell numbers were normal compared to healthy control dogs. The few remaining β-cells appeared swollen, vacuolated, and degranulated[110]. Additionally, diabetic islets were poorly defined compared with controls and inflammatory mononuclear islet infiltrates were observed in 6 out of 13 dogs[102,110]. Despite the several possible causes of canine diabetes, the fact that there are variations in the prevalence of this disease among different breeds strongly suggest that genetic factors contribute to susceptibility to the disease[109]. However, to date there is no reported association between breed/genetics and autoimmune markers such as autoantibodies in diabetic dogs[110]. Some types of genes associated with canine diabetes are similar to those seen in humans, indicating that specific genes influencing disease susceptibility may be shared between species[109]. Environmental factors such as viruses, dietary elements, or toxins that have been implicated in the pathogenesis of T1D in humans (acting as both triggers and enhancers of β-cell destruction) are also presumed to play a role in the etiopathogenesis of canine diabetes[114].

Regardless of the various underlying mechanisms that can cause canine diabetes, they typically result in a similar set of clinical signs associated with hyperglycemia already described for human diabetes. The hallmark of diabetes is osmotic diuresis, leading to polydipsia and polyuria. It’s also common to find weight loss, and sometimes polyphagia. If left untreated, the condition may progress to more severe symptoms such as inappetence, lethargy, vomiting, and in some cases, the development of diabetic ketoacidosis[108].

2.2. Feline diabetes

Although there are no documented cases of T2D in dogs, approximately 80% of diabetic cats exhibit insulin-independent diabetes. However, it's worth noting that T1D can also manifest in cats[98].

Like human T2D, feline diabetes is a heterogeneous condition resulting from a combination of impaired insulin action in the liver, muscle, and adipose tissue (insulin resistance), and progressive β-cell failure[98]. On average, diabetic cats are six times less sensitive to insulin compared to non-diabetic cats[104].

The progression of feline diabetes closely mirrors that of T2D in humans[115]. Insulin resistance in peripheral tissues generates a chronic high demand for insulin secretion, as higher plasma insulin concentrations are required to achieve the same glucose uptake into tissues compared to normal insulin sensitivity[104]. As insulin resistance advances, glucose clearance becomes disrupted even during fasting, leading to continuous irregular insulin secretion[116]. This can result in oxidative stress and increased apoptosis in the Langerhans islets, ultimately leading to progressive β-cell failure[104]. Additionally, hypersecretion of insulin leads to increased secretion of the islet amyloid polypeptide hormone leading to amyloid deposition in the Langerhans islets[116]. Once diabetes is established, the continued hyperglycemia itself contributes to β-cell dysfunction[117]. Pancreatitis is evident histologically in approximately 50% of diabetic cats and may also contribute to β-cell dysfunction[104]. The proposed progression of events leading to feline diabetes is illustrated in Figure 4.

Both environmental and genetic factors are believed to contribute to the development of feline diabetes[98], including genotype, obesity, physical inactivity, diet, exposure to toxic chemicals and drugs that cause insulin resistance[104,118].

The fact that male cats are more predispose to diabetes compared to female cats may be due to gender differences in weight gain and insulin sensitivity[119]. While increased weight is a known risk factor for diabetes in both genders, a study found it to be significant only in male cats. Also, precious studies have established that male cats are more prone to weight gain[120] and have higher basal insulin levels and lower insulin sensitivity[121], suggesting a natural predisposition to insulin resistance. As male cats gain weight, their insulin sensitivity decreases, and insulin levels rise. They also tend to accumulate more fat than female cats, partly because they exhibit greater glucose oxidation, glycogenesis, and lipogenesis in response to insulin. These metabolic differences help explain why male cats a higher risk of developing diabetes have compared to female cats[118].

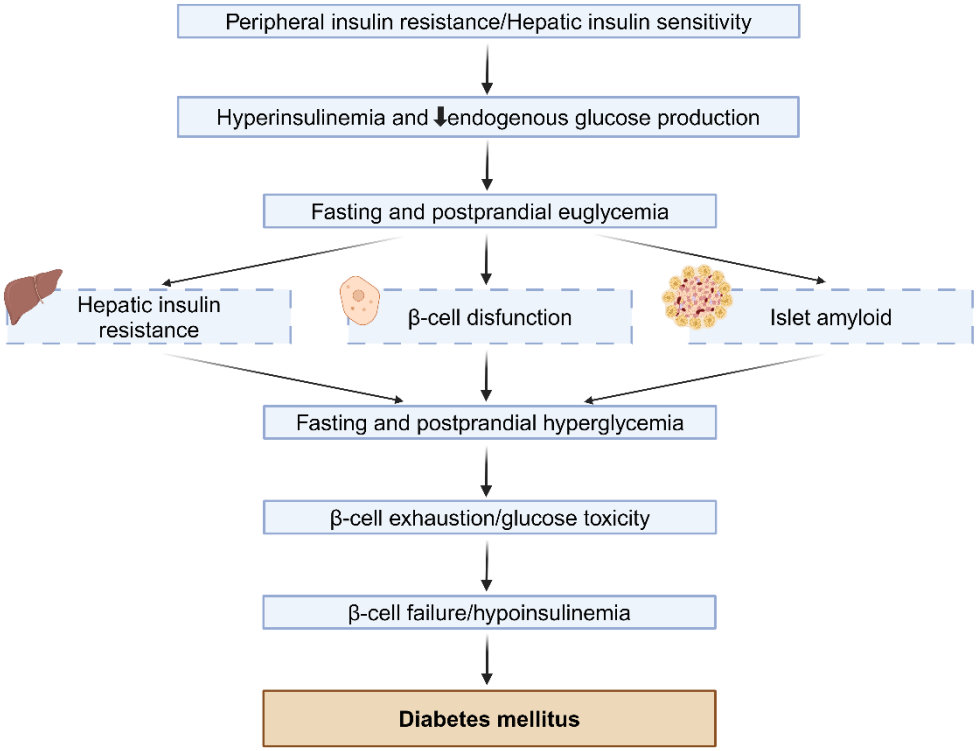

Figure 4 - Sequence of events in the progression from obesity to diabetes in cats, shedding light on the cascade of physiological changes and molecular mechanisms underlying this transition.

Like what's happening in human medicine, the prevalence of feline obesity is raising as up to 40% of feline are obese or overweight and so is the prevalence of diabetes[116]. Obesity, together with physical inactivity, are believed to be the main contributors to the insulin resistance associated with diabetes in cats regardless of gender[117,119]. In one study, cats that gained 2 kilograms over ten months experienced a 50% decrease in insulin sensitivity. In average, each excess kilogram of body weight results in a 30% decline in insulin sensitivity[117]. Additionally, urban cats, especially those confined indoors without the opportunity to hunt, are less physically active than feral cats and are more likely to develop insulin resistance[104]. They are usually fed commercial diets high in fat elevate fatty acid concentrations, often in abundancy, which further diminish insulin sensitivity[104,119]. Additional factors contributing to excess body weight in cats include the owner's behavior, such as giving treats and using food to bond with the pet and neutering. Neutered cats are also more prone to becoming overweight if their caloric intake is not regulated, as gonadectomy reduces energy requirements and increases voluntary food consumption[117].

Drugs such as corticosteroids and progestins can decrease insulin sensitivity, particularly with long-term or repeated use. These medications also increase appetite, leading to weight gain and further reducing insulin sensitivity[104].

As mentioned previously, albeit uncommonly, cats can also exhibit a form of diabetes akin to T1D in humans. One of the underlying mechanisms involves immune-mediated lymphocyte infiltrations within the islets but, unlike in dogs, the presence of β-cell and insulin antibodies have not yet been demonstrated in diabetic cats[122]. Nonetheless, a genetic predisposition has been proposed in Burmese cats, which exhibit a significantly elevated incidence of diabetes. However, the specific gene or genes potentially implicated in this predisposition have yet to be identified[114].

Diabetic cats exhibit typical signs of diabetes, such as polydipsia and polyuria. Polyphagia can be difficult for owners to assess, as many cats had a high appetite even before developing diabetes. Weight loss is not always reported[104].

2.3. Feline and canine diabetes and the GI tract

There are almost no reports of GI changes in diabetic pets. Accordingly, to Kelly Diehl "although there is considerable information regarding gastrointestinal complications of diabetes in humans, there is little more than anecdotal information about these problems in dogs and cats"[123]. However, diabetes has been suggested as one of the possible causes of GI dysmotility in critically ill dogs and cats[124] and a thickened small intestine has been associated with intestinal impaction in diabetic dogs[125]. In a review of cases of diabetes seen at the Colorado State University Veterinary Teaching Hospital about 38% of those dogs and 31% of cats also had GI disease[123].

There is only one paper focused on the GI tract of diabetic dogs, that examined the impact of spontaneous diabetes on the nitrergic neurons within the myenteric plexus of both the canine gastric antrum and ileum. The results unveiled a notable reduction in the density of nitrergic neurons in both antrum and ileum of diabetic dogs compared to control group. Additionally, diabetic dogs exhibited a thickening of the periganglionic connective tissue surrounding the ganglia. The study concluded that diabetes in dogs leads to significant alterations in the myenteric neuronal composition, particularly affecting the nitrergic neuronal subpopulation[126].

3. Animal models of Diabetes

Given the widespread prevalence and significant impact of diabetes, alongside the indispensable role of animal models in advancing scientific knowledge, the significance of utilizing such models for studying the disease's etiology and testing novel antidiabetic medications becomes evident[127]. Animal models serve as invaluable tools in unraveling the underlying mechanisms of diabetes and evaluating the efficacy of potential treatments,

thereby facilitating progress in diabetes research and therapeutic development[128]. A recent report on toxicological studies highlighted that there is a significant 71% concordance between adverse findings in clinical data and those observed in experimental animal models. This high level of agreement underscores the reliability and relevance of animal models in predicting human diabetic complications[129]. There are several animal models of diabetes in different laboratory animal species, which include surgical (pancreatectomy), chemical or genetic models[130]. The majority of the diabetes studies are performed in rodents (mice and rat) although there are some investigations in larger animals[131].

Experimental animal models are classified according to the type of diabetes they mimic. T1D is usually induced in experimental animals either through chemical destruction of pancreatic β-cells or by breeding rodents that spontaneously develop autoimmune diabetes[132]. In contrast, T2D models are more diverse, encompassing both obese and non-obese models with varying degrees of insulin resistance and β-cell failure[131].

3.1. The Streptozotocin-Induced Experimental Model of diabetes

Chemical models are one of the most common methods for inducing diabetes and generally use streptozotocin (STZ) or alloxan as the trigger (69% and 31% of the published studies, respectively)[133].

STZ, chemically known as N-(methylnitrosocarbamoyl)-α-d-glucosamine, is a naturally occurring compound synthetized by *Streptomyces achromogenes*, with antimicrobial and chemotherapeutic properties[131,134]. This compound has been the agent of choice to induce diabetes since 1963, because of its ability to induce structural, functional and biochemical alterations that resemble those seen in diabetes[135]. The induction of diabetes with STZ is most frequently used in rats and mice, but has also proven to be efficient in rabbits, hamsters, guinea pigs, pigs and gerbils[73,130,136]. The diabetogenic effect of STZ is due to the selective destruction of pancreatic β-cells. These cells are in constant need for glucose, uptake via insulin-independent GLUT-2 transporters that are abundant on these cell plasma membranes[137]. After intraperitoneal or intravenous administration of STZ, this compound uses GLUT-2 transporters to enter pancreatic β-cells, causing their destruction[136]. STZ toxicity is multifactorial but occurs through three main mechanisms. First, STZ intracellular accumulation causes DNA alkylation, resulting in cell necrosis. Then, STZ itself acts as a nitric oxide donor, which is known to cause damage in pancreatic β-cells DNA. In addition, STZ produces ROS like superoxide that accelerate β-cell destruction through disruption of ATP production in the mitochondria[130,135]. This DNA damage triggers the activation of poly ADP-ribosylation, which reduces intracellular NAD^+ levels and further decreases the available ATP in the mitochondria. This overall reduction in ATP leads to diminished insulin

synthesis and secretion from the β-cells (figure 5). The resulting glucose overload produces downstream glycation end products, which exacerbate oxidative stress, leading to continued cell apoptosis, necrosis, and additional DNA damage[138].

STZ can be used to cause both T1D or T2D, depending on if it is administered as a single high dose or as multiple low doses[132]. At high doses, STZ causes massive destruction of β-cells resulting in an insulin production deficiency that causes T1D (figure 5A). Administration can be performed via intravenous or intraperitoneal routes, with dosages ranging from 100 to 200 mg/kg in mice or 35 to 65 mg/kg in rats[131]. At lower doses, typically administered as multiple exposures, STZ induces an immune and inflammatory response, characterized by inflammatory infiltrates, particularly lymphocytes, in the pancreas, leading to a hyperglycemic state (figure 5B). Various low-dose STZ protocols suggest administering small doses, such as 20 to 40 mg/kg/day. This method can also be used in association with a high-fat diet[129].

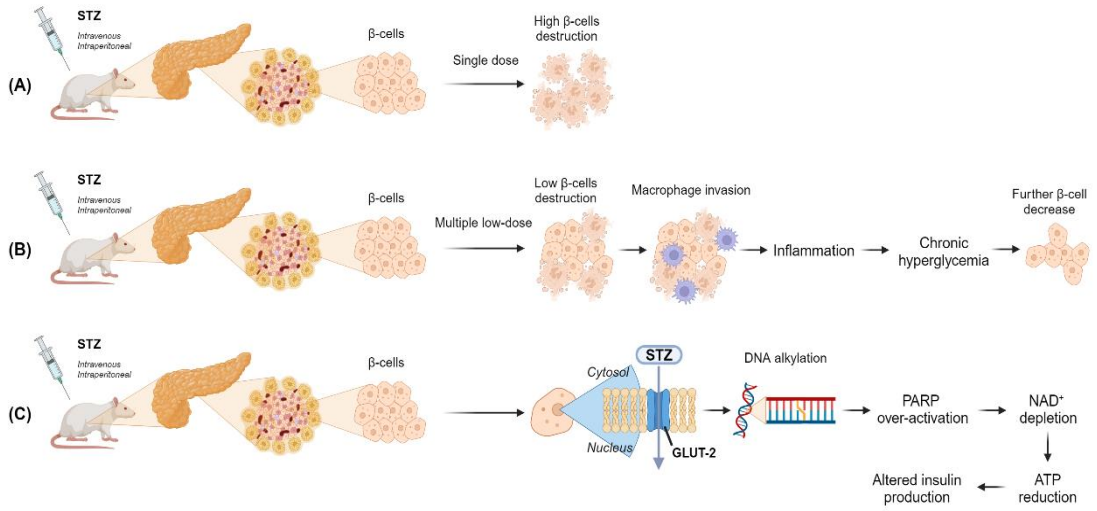

Figure 5– Streptozocin (STZ) induction of diabetes model. (A) Single-dose STZ causes β-cells destruction and type 1 diabetes; (B) Multiple-low dose STZ causes β-cells decrease and type 2 diabetes, (C) STZ mechanism of action on the β-cells nucleus; PARP-poly-ADP ribose polymerase.

Susceptibility to the diabetogenic effects of STZ decreases with age and is higher in males than in females. Wistar and Sprague-Dawley are the rats' strains more sensitive to STZ, whereas certain other strains, such as Wistar-Kyoto rats, exhibit reduced sensitivity[129].

STZ is considered a selective drug, since it does not affect pancreatic parenchyma or α-cells but may cause injury in other cells that express GLUT-2 transporters, like hepatocytes and renal tubular cells. For this reason, some authors defend that this model is not recommended to study renal or hepatic effects of diabetes[130] but this is debatable. Other

authors believe that due to the short life of STZ (15 minutes) and rapid metabolization in the liver and elimination the kidneys, the acute toxicity in these organs can be neglected. Therefore, after STZ is eliminated out of the body, any further functional damage of the liver and the kidney may be attributed to the effects of diabetic hyperglycemia [137].

3.2. The Goto-Kakizaki model of diabetes

The Goto-Kakizaki (GK) rat model is a widely used and is considered one of the best non-obese models for studying T2D[139]. This model was developed in the 1970s by Dr. Goto, Dr. Kakizaki and colleagues through selective breeding of Wistar rats with naturally glucose intolerance[140,141]. Over generations, the amplification of the diabetic traits led to a strain that spontaneously exhibits many characteristics of human T2diabetes and the establishment of the GK rats of a model of T2diabetes[142].

The glucose intolerance in GK rats is suggested to be primarily caused by impaired insulin secretion due to defective β-cell function[143]. This is supported by the fact that the Langerhans islets of GK rats exhibit decreased β cell mass, altered islet architecture, and reduced insulin content[144]. In fact, in adult GK rats, the total pancreatic β cell mass and insulin stores are reduced by 60% due to a significant decrease in β cell replication[145]. Additionally, GK rats' pancreatic islets have disrupted architecture, with fibrosis separating strands of endocrine cells, giving them a starfish-like appearance. These changes are not seen in young GK rats but become more common as the rats age[146]. Besides the reduced pancreatic β-cell function and number, the animals present moderate hyperglycemia, post prandial glucose intolerance and peripheral insulin resistance (figure 6)[147]. Chronic hyperglycemia causes glucotoxicity and can further impair β cell function and insulin action, contributing to hyperglycemia progression [146].

Unlike many other animal models of T2D that rely on inducing the disease through diet or drugs, which often result in obesity, GK rats develop diabetes without becoming obese[148]. This unique characteristic makes GK rats particularly valuable for studying diabetes and diabetes related complications such as nephropathy, neuropathy, and cardiovascular diseases independently of obesity[149].

The hereditary nature of the condition in GK rats closely mimics the genetic predisposition observed in humans. This similarity allows for more accurate studies on the genetic factors contributing to T2D, providing insights that are more directly applicable to human diabetes research. Consequently, the GK rat model serves as a crucial tool for exploring the genetic underpinnings of T2D and developing targeted treatments that address the disease at its root cause[131]. One example of the similarities with T2D is that exercise can help moderate hyperglycemia in GK rats by increasing the muscles' ability to capture circulating glucose

due to reduced intramuscular fat. After a training period, running GK rats showed significantly lower fasting blood glucose levels[146]. However, while the GK rat model replicates many aspects of human T2D, there are differences in disease progression and manifestation between rats and humans. The environmental and dietary factors influencing human T2D are not fully replicated in this model. Also the early β-cell destruction is not usually observed in non-obese type 2 diabetic humans[150].

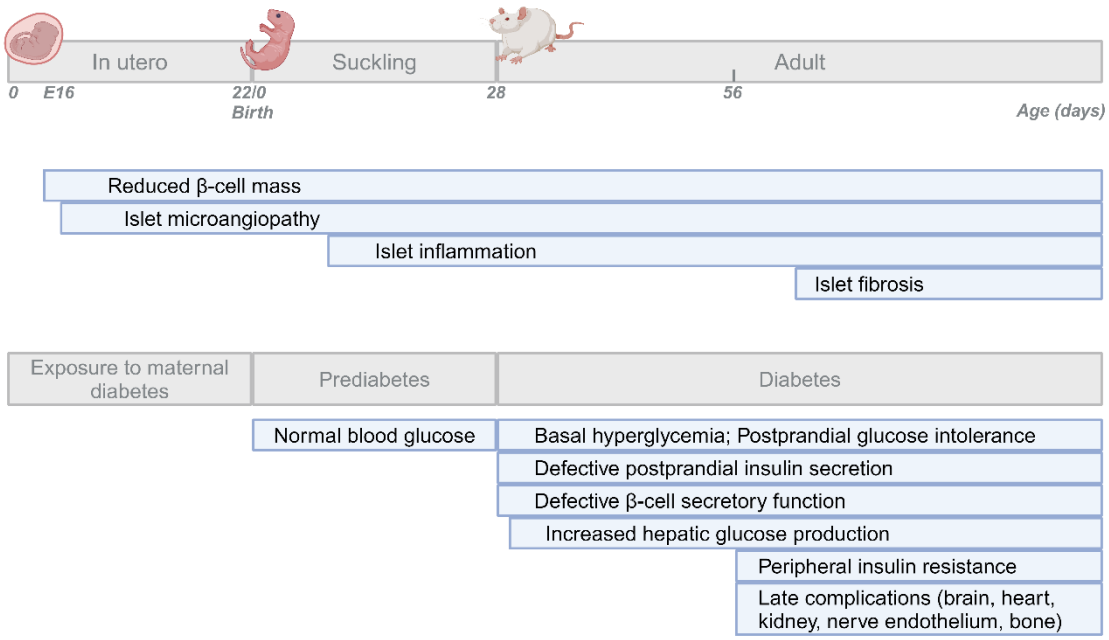

Figure 6 -Time-course of the development of diabetes in Goto-Kakizaki (GK) rats. Increased glucose production and decreased insulin sensitivity have been reported in the liver at early stage (weaning). In adult GK rats, plasma insulin release in response to glucose administration is decreased. Moderate insulin resistance in extrahepatic tissues (muscles and adipose tissues) develops later, with complications of long-standing diabetes. Hyperglycemia is preceded by a period of normoglycemia, ranging from birth to weaning. Islets from adult GK are infiltrated by inflammatory cells and their vascularization is altered. Islet fibrosis becomes prominent with aging.

3.3. The 3Rs Principles in research

Animal models are crucial for advancing scientific knowledge, and when experimental work involving animals is conducted, adhering to the 3Rs principles becomes essential. The 3Rs principles in research - Replacement, Reduction, and Refinement - are ethical guidelines aimed at ensuring the humane treatment of animals used in scientific research[151]. These principles were first proposed by Russell and Burch in their 1959 book "The Principles of Humane Experimental Technique"[152].

Replacement refers to methods that avoid or replace the use of animals in research. This can be achieved through several approaches, such as the use of cell cultures, computer models, or advanced imaging techniques[153].

Reduction involves strategies to minimize the number of animals used in experiments. This can be accomplished by optimizing experimental designs and statistical analyses to ensure that the smallest number of animals is used to obtain valid results [154]. Techniques such as organs and tissue sharing between different experimental groups can help in reducing the number of animals required since it's possible to obtain more information from the same animals[155].

Refinement refers to methods that enhance animal welfare and minimize or eliminate pain, suffering, and distress[151]. This principle focuses on improving the conditions under which animals are housed and cared for, as well as refining experimental procedures to make them less invasive or stressful[156]. Examples include the use of analgesics and anesthetics to manage pain, providing environmental enrichment to promote natural behaviors, and training animals to cooperate with procedures to reduce stress[157].

More recently, a fourth 'R' has been introduced to the ethical framework of animal experimentation, representing 'Responsibility.' This addition emphasizes the growing recognition of researchers' duty to ensure that all animal experiments are conducted with the highest ethical standards, reinforcing the commitment to minimize harm and prioritize animal welfare[158].

By adhering to the 3Rs principles, researchers aim to conduct ethical and humane scientific investigations while still achieving robust and reproducible results. These principles not only promote the welfare of animals but also improve the quality of scientific research by ensuring that animal use is justified and optimized[151].

In Europe, compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes is mandatory. This legislation sets stringent guidelines to ensure the ethical treatment and welfare of animals in scientific research, emphasizing the principles of the 3Rs. In Portugal, Decree-Law No. 113/2013 implements the EU Directive 2010/63 on the protection of animals used for scientific purposes. All research projects involving animals must receive approval from the ORBEA (Organismo Responsável pelo Bem-Estar Animal) and the DGAV (Direção-Geral de Alimentação e Veterinária) to ensure compliance with ethical and legal standards.

4. The Renin Angiotensin Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) is a key regulator of cardiac, vascular, and renal function. It consists of enzymes and peptides that control blood pressure by regulating vasoconstriction, sodium reabsorption, and body fluid balance[159]. Although the RAAS was initially discovered over a century ago[160], it continues to be extensively studied,

with new peptides and enzymes still being identified. Current research suggests that the RAAS operates through two distinct pathways: the classic arm and the counter-regulatory arm[161].

In the classical view, the activation of renal juxtaglomerular cells in the afferent arterioles of the kidneys triggers the conversion of prorenin to renin[162]. Renin is released into circulation when there is decreased in arterial pressure and decreased renal perfusion, reduced tubular sodium content, and sympathetic stimulation of the β-adrenergic system, being considered the rate-limiting enzyme in the RAAS[163]. Angiotensinogen, primarily synthesized and secreted by the liver, is cleaved by renin in the N-terminal fragment into angiotensin I (Ang I), which has no known biological activity but serve primarily as a substrate to produce other biologically active peptides[162]. Angiotensin I is then converted to angiotensin II (Ang II) by the pulmonary Angiotensin-Converting Enzyme (ACE) that cleaves the C-peptide[163,164]. Ang II is the primary mediator of the physiological effects of this system, causing vasoconstriction, aldosterone release, promoting inflammation and fibrosis, amongst other effects (figure 7)[163]. Aldosterone, a hormone produced by the adrenal cortex, is essential for maintaining water and electrolyte balance[162]. Its main function is to increase sodium and water retention, which raises blood volume. This process occurs by activating mineralocorticoid receptors in the kidney's distal tubules, leading to increased sodium transport across cell membranes[165].

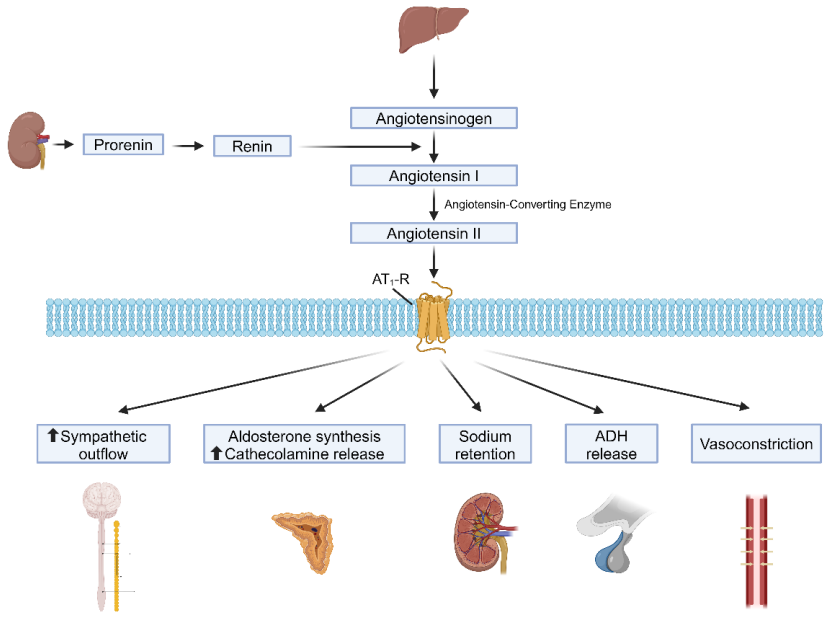

Figure 7 - The activation of systemic renin-angiotensin begins with renin secretion by the kidney. Once it has been released into the bloodstream, renin cleaves angiotensinogen to form angiotensin I, which is then converted to angiotensin II (Ang II) by pulmonary angiotensin converting enzyme. Ang II stimulates vasoconstriction, renal retention of salt and water, aldosterone secretion, and sympathetic activity, whereby it increases blood pressure.

The physiological effects of Ang II, as well as the pathophysiological effects (which will be discussed further), are mediated by two types of receptors: type 1 (AT_1R) and type 2 (AT_2R). These receptors elicit different and often opposing physiological responses (figure 8)[164]. Both AT_1R and AT_2R are G-protein coupled receptors. AT_1R is widely distributed across many cell types in the body[163]. The interaction of angiotensin II with this receptor is responsible for its physiological effects, such as vasoconstriction and sodium and water reabsorption, as well as pathological effects including inflammation, fibrosis, oxidative stress, tissue remodeling, and increased blood pressure (figure 8)[159]. In contrast, AT_2R is less widely distributed, and its expression decreases in adulthood[163,166]. Despite its low expression levels in adults, AT_2R mediates the opposing effects of angiotensin II, counteracting the actions of AT_1R. This receptor mediates several effects such as vasodilatation, antiproliferative and anti-fibrotic actions[166,167].

The view of the RAS has been expanding with additional truncated peptides such as Ang 1 -7[167,168], enzymes like serine protease chymase (which can also cleave ang I to form Ang II)[169] and receptors (like AT_4)[170] identified. Angiotensin converting enzyme 2 (ACE2) degrades Ang II to generate Ang 1-7[171], which appears to exert multiple actions, predominantly antagonistic to those attributed to Ang II[172].

Furthermore, the traditional view of the RAAS as a circulating hormonal system has evolved to include an understanding that, in addition to the circulating RAS, there exists a local tissue RAAS[159]. It was observed that many tissues can synthesize key components of the RAAS, containing all components necessary to produce Ang II[173]. For instance, in the heart, renin, angiotensinogen, ACE, and Ang II receptors are all present[174], and the local RAAS is upregulated in response to injury[159]. Interestingly, the circulating and local systems can exhibit contrasting behaviors[175].

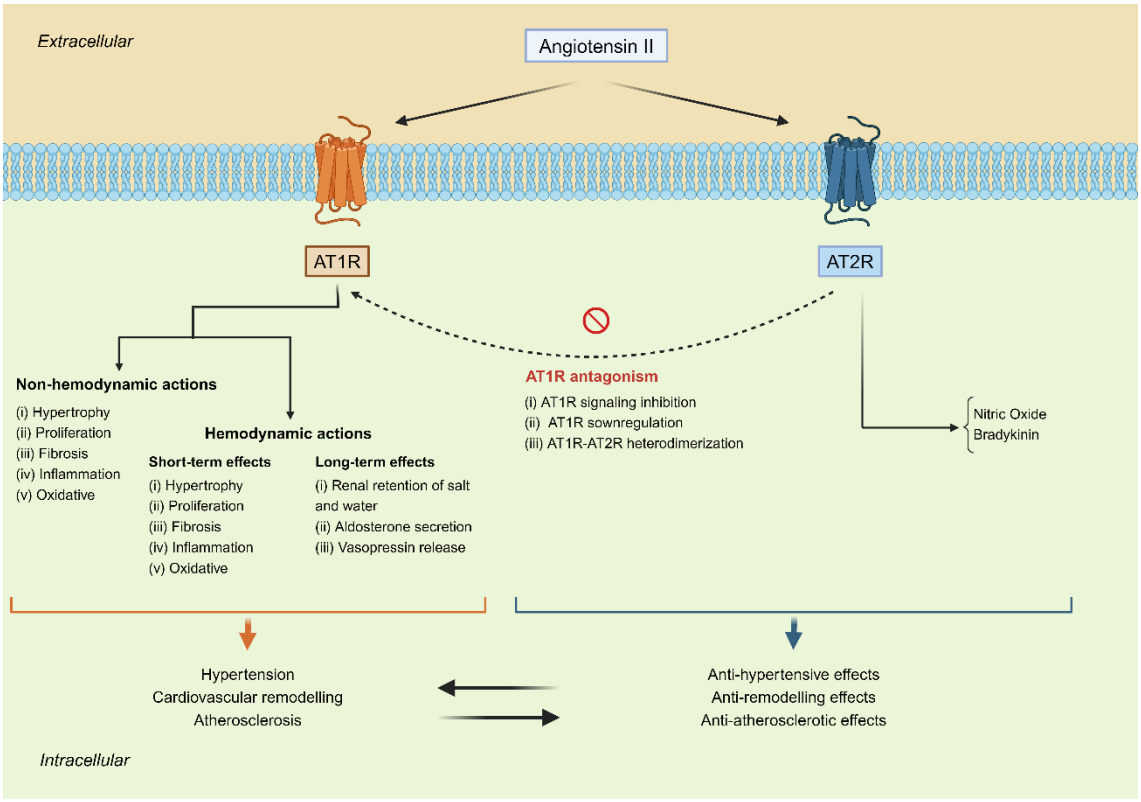

Figure 8 - Angiotensin II has two major receptor isoforms: AT_1R and AT_2R. AT_1R stimulation mediates the classical actions of Angiotensin II, including nonhemodynamic effects. On the other hand, AT_2R stimulation usually causes opposing effects to AT_1R. Moreover, it can antagonize AT_1R by downregulating it, inhibiting its signaling or binding to it.

4.1. ACE and ACE2

ACE was first discovered in the 1950s when researchers noticed that treating plasma and kidney extracts with water and saline produced two substances that raised blood pressure: Ang I and Ang II. In 1966, ACE was found again when researchers identified an enzyme in the kidney that broke down bradykinin, a substance involved in lowering blood pressure. This enzyme, initially called kininase II, turned out to be the same as ACE. ACE2, a similar enzyme, was discovered in 2000[176].

ACE is a membrane-bound zinc metalloprotease that consists of a large polypeptide chain, approximately 1300 amino acids in length. It is a type I transmembrane protein, characterized by a single transmembrane helix that anchors it to the cell membrane, with a large extracellular domain that houses its enzymatic activity[177]. The extracellular portion of ACE can be divided into two distinct catalytic domains: the N-domain and the C-domain, both of which contribute to its overall function but have some functional differences. ACE also possesses a C-terminal transmembrane segment[178].

The C-terminal ACE catalytic domain is responsible for maintaining a functional renin-angiotensin system, being responsible for most angiotensin II production as it has a high

affinity for Ang I[179] and the degradation of bradykinin[180]. Bradykinin is a peptide that typically induces vasodilation and lower blood pressure, so the C-domain's ability to degrade it helps prevent excessive vasodilation, thereby maintaining blood pressure homeostasis[181]. In addition to bradykinin, the C-domain also has the ability to cleave peptides such as enkephalins and substance P, although it is most efficient at hydrolyzing Ang I and bradykinin[179].

The N-domain shares a similar structural design to the N-domain but exhibits some important differences in functionality. While the N-domain also contributes to the conversion of Ang I into Ang II, it does so less efficiently and with lower affinity compared to the C-domain[182]. The N-domain is particularly adept at processing specific substrates, such as the tetrapeptide N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP). Ac-SDKP is a naturally occurring peptide that functions as an anti-fibrotic and anti-inflammatory agent. By degrading Ac-SDKP, the N-domain influences fibrotic and inflammatory processes[183]. Due to this hydrolyzation of the antifibrotic agent Ac-SDKP, N-domain selective (N-selective) inhibitors are considered as potential treatments of conditions relating to excessive tissue fibrosis[184]. Additionally, the N-domain's capability to degrade Ac-SDKP is significant in hematopoiesis, as Ac-SDKP inhibits the proliferation of hematopoietic stem cells[182]. Like the C-domain, the N-domain also degrades bradykinin, contributing to the regulation of vascular tone. The N-domain also metabolizes Ang 1-5 and Ang 1-7 which seems to have cardioprotective effects[182].

Both catalytic domains are zinc metallopeptidases, where two histidine residues coordinate the zinc ion. Hip-His-Leu (h-HL) is usually used as a substrate for the C-domain due to its high affinity being cleaved much more efficiently by the C-terminal domain[179], while benzyloxycarbonyl-phenylalanyl-histidyl-leucine (Z-FHL) is commonly used as a substrate for both domains[176].

ACE2, a homolog of ACE sharing 40% similarity, is a chimeric protein with a single catalytic domain from ACE (C-domain) and a C-terminal domain[176]. Contrary to ACE, ACE2 does not degrade bradykinin and it's not inhibited by ACE inhibitors. ACE 2 has garnered attention due to its role in degrading Ang II and generating Ang 1-7, a peptide that counteracts some of the effects of Ang II, including vasodilation and antifibrotic properties. ACE2 can also metabolize Ang I to produce Ang 1-9, which is subsequently cleaved by ACE to generate Ang 1-5. However, ACE2 has a higher affinity for Ang II than for Ang I, and its primary function is the degradation of Ang II[185]. ACE2 has also gained prominence because of its involvement in the entry of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) into cells, as the virus uses ACE2 as a receptor for cell entry[186].

The ACE N- and C-domain ratio and the ACE2/ACE ratio are important for understanding the delicate balance of the RAAS and the regulation of homeostasis. The possible values

of the N/C-domain ratio are still under investigation, but it is believed that the N-domain contributes to a significantly larger portion of the enzyme's overall activity[179]. While exact values can vary depending on tissue type and individual genetic factors, studies suggest that in most tissues, the N-domain is responsible for most of the ACE activity, and the ratio of N to C activity could range from about 3:1 to 10:1[187,188]. This can vary depending on the specific physiological or pathological context[179]. The ACE2/ACE ratio represents the balance of two enzymes with opposing effects in the RAAS. A higher ACE2/ACE ratio promotes the generation of Ang1-7, which has opposing effects to Ang II, such as vasodilation, anti-inflammatory effects, and cardioprotection[185]. ACE2 is also important in counterbalancing the negative effects of ACE, including its role in inhibiting fibrosis and protecting the kidneys[189]. A lower ACE2/ACE ratio ends to favor the production of Ang II, leading to increased vasoconstriction and higher blood pressure. This is often observed in conditions such as hypertension, heart failure, chronic kidney disease and diabetes where overactivation of the ACE pathway can contribute to disease progression and organ damage[175,185,189–191].

4.2. Pathophysiological role of RAAS

One of the first potential negative effects of dysregulation of the RAAS are hypertensive disorders[166]. However, it has been clearly shown that the Ang II effects occur independently from its effects on blood pressure and that the RAAS plays a pivotal role in several non-hypertensive conditions[192]. It seems that Ang II plays a part in the pathogenesis of chronic fibrogenetic diseases of various organs, including kidney, heart, lung, pancreas and liver, through the regulation of both inflammatory and fibrotic processes[164]. However, it is important to note that the role of the RAAS in these disorders has traditionally been understood in terms of the classical circulating RAAS, while the potential contributions of tissue RAAS dysregulation are still being elucidated in several organs[166]. Besides Ang II effects, research also shows that elevated levels of aldosterone can directly cause inflammation and fibrosis, contributing to the damage of organs such as the blood vessels, kidneys, and heart, resulting in conditions like vasculitis, fibrosis, and organ hypertrophy[166,193].

In general, fibrosis usually occurs as a response to injury. The initial phase, known as the inflammatory phase, immediately follows the insult and involves the activation of the coagulation cascade, fibrin deposition, and the infiltration of macrophages and neutrophils[194]. This is followed by the proliferative phase, which is defined by angiogenesis, fibroblast proliferation, and differentiation. Finally, the remodeling phase involves fibroblasts and myofibroblasts depositing a collagen-rich extracellular matrix (ECM), forming a scar

that replaces the damaged functional tissue[195]. Chronic tissue injury can also lead to fibrosis, where the normal wound healing response becomes persistent and pathological. This condition is characterized by chronic inflammation and the continuous presence of myofibroblasts, resulting in excessive ECM accumulation and the disruption of normal tissue architecture[196]. In response to ongoing tissue damage, fibroblasts produce large amounts of ECM proteins and express α-smooth muscle actin (α-SMA), which reduces tissue compliance. The activation of fibroblasts and myofibroblasts is regulated by various soluble factors, including cytokines, growth factors, and oxidative stress products[197]. Among these, transforming growth factor-beta 1 (TGF-β1) is particularly crucial in triggering and sustaining the fibrogenesis process[198]. Fibrosis leads to tissue remodeling, resulting in decreased tensile strength of the affected tissue. The fibrotic tissue also shows reduced cell density and metabolic activity[199]. Additionally, there are changes in the type, amount, and organization of collagen. Initially, type III collagen is synthesized at high levels but is eventually replaced by type I collagen. Healed or repaired tissue never regains the strength of normal, uninjured tissue[194].

But how is this related to RAAS? Locally, Ang II binding to AT_1R activates multiple intracellular signaling pathways that stimulate profibrotic downstream effects, namely inflammatory cell recruitment, angiogenesis, cellular proliferation, and accumulation of ECM in several organ systems in the organism, especially cardiovascular and renal systems[195]. This includes the activation of mitogen-activated protein kinases (MAPKs), Janus kinase/signal transducers and activators of transcription (JAK/STAT), and nuclear factor kappa B (NF-κB) pathways, all of which contribute to the synthesis of ECM components[200]. Furthermore, Ang II is a promotor of inflammation due to the stimulation of the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β). These cytokines recruit inflammatory cells to the site of injury, perpetuating a chronic inflammatory state that is conducive to fibrosis[201].

In addition to these actions, the key mediators of fibrosis induced by Ang II are TGF-β1 and ROS expression (figure 9)[164,202].

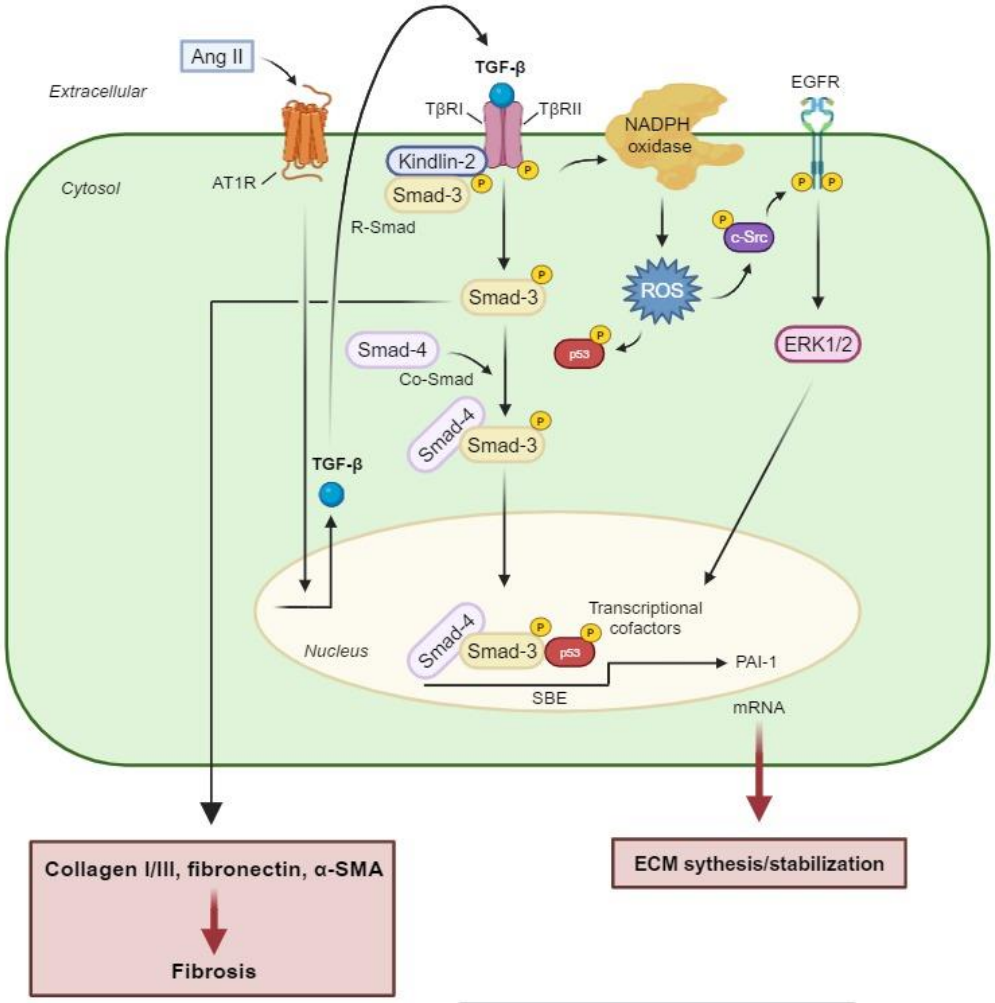

Figure 9- Transforming growth factor (TGF)-β1 and reactive oxygen species (ROS) are key players in angiotensin II (Ang II)-induced fibrosis. Ang II increases the expression of TGF-β1. TGF-β1 binds to its receptor (TbRII), which activates another receptor (TbRI) that phosphorylates Smad3. Kindlin-2 assists in the interaction between TbRI and Smad3, enhancing Smad3 activation. The phosphorylated Smad3 then combines with Smad4 to form a complex that moves into the nucleus and binds to specific DNA regions, activating target genes like plasminogen activator inhibitor-1 (PAI-1) and certain microRNAs (miRNAs). Additionally, TGF-β1 generates ROS, which activate the epidermal growth factor receptor (EGFR) and p53. These molecules interact with phosphorylated Smad3 and other cofactors to sustain gene activation. TGF-β1 promotes fibrosis through Smad3.

TGF-β1 is a master regulator of fibrosis[198]. Ang II stimulates the expression and activation of TGF-β1, which in turn promotes fibroblast activation, ECM production and deposition, and inhibition of ECM degradation. TGF-β1 signaling involves both Smad and non-Smad pathways for the activation of its major effects (figure 9)[202]. The proliferation of fibroblasts and their differentiation into myofibroblasts are responsible for the excessive deposition of ECM proteins, such as collagen. In these conditions, synthesis of collagen types I and III is upregulated, leading to an imbalance between collagen synthesis and degradation, favoring

ECM accumulation[203]. Myofibroblasts express α-SMA, which enhances their contractile function and contributes to tissue stiffening and decreased compliance[202]. Furthermore, the inhibition of Matrix Metalloproteinases (MMPs) activity by Ang II, which are enzymes responsible for ECM degradation, further contributes to the excessive accumulation of ECM components[204].

Oxidative Stress is responsible for direct cellular damage and also has the potential to further stimulate inflammatory and fibrotic pathways[202]. Ang II can increase the production of ROS through the activation of NADPH oxidase, an enzyme complex that transfers electrons from NADPH to oxygen, forming superoxide (figure 9a)[205]. Ang II can also induce mitochondrial ROS production, leading to further oxidative stress and cellular damage[206]. ROS contributes to fibrosis through several mechanisms. Firstly, there is an interplay with TGF-β1, which, as mentioned earlier, can increase ROS production. Simultaneously, ROS enhance the activation of TGF-β1 and the TGF-β1/Smad signaling pathways, creating a feedback loop that amplifies fibrosis[207]. ROS also act as secondary messengers in various signaling pathways, activating kinases and transcription factors that lead to the expression of pro-fibrotic genes[208]. Elevated ROS levels can upregulate MMPs, contributing to ECM remodeling and fibrosis[209]. Additionally, ROS promote inflammation by activating inflammatory cells (e.g., macrophages) and inducing the release of pro-inflammatory cytokines (e.g., TNF-α, IL-6) which is a known driver of fibrosis[208].

4.3. Pharmacological inhibition of RAAS

AT_1R is upregulated following injury in various tissues, including the heart, blood vessels, brain, nerves, and skin. Enhanced Ang II signaling through AT_1R in injured tissues is well-documented in cardiovascular and renal diseases, as well as in Alzheimer's disease, stroke, and several other conditions[195]. For this reason, the pharmacological inhibition of the RAAS has shown to be crucial therapeutic strategy for treating hypertension, heart failure, chronic kidney disease, cardiovascular disorders and other conditions [163]. The pharmacological inhibition of the RAS targets several pathways: (1) inhibiting Ang II generation with ACE inhibitors (ACEIs); (2) antagonizing Ang II actions with AT_1R blockers (ARBs); (3) inhibiting the conversion of angiotensinogen to Ang-I with direct renin inhibitors (DRIs); and (4) using aldosterone receptor antagonists (Mineralocorticoid Receptor Antagonists - MRAs) (figure 10)[210].

DRIs, such as Aliskiren block renin activity, blocking the conversion of angiotensinogen to angiotensin I (figure 10). By inhibiting renin, these drugs reduce the formation of angiotensin I and consequently angiotensin II[211]. However, this medication has modest side effects, and its effectiveness in lowering blood pressure and protecting against end-organ damage in

patients with hypertension, chronic heart failure, type 2 diabetes, and chronic kidney disease is comparable or inferior to ACEIs and ARBs[212–215]. Additionally, Aliskiren has been shown to cause a higher reactive rise in plasma renin levels compared to ACEIs and ARBs, potentially activating fibrotic signaling pathways via the renin receptor[216]. Furthermore, the proximal blockade of the RAS may reduce the production of protective angiotensin peptides, such as Ang-1-7[217]. For these reasons, this medication is not commonly used[210].

ACEIs were initially used to treat refractory hypertension. However, their significance has grown due to their beneficial effects in reducing morbidity and mortality in conditions such as congestive heart failure, myocardial infarction and chronic renal insufficiency[218]. The first ACE inhibitor was captopril, which had many unacceptable side effects. This was followed by the development of several other drugs, including lisinopril, ramipril, enalapril, fosinopril, and benazepril[166]. Nowadays, ACEIs are considered a first-line treatment for hypertension and other conditions because they prevent the production of Ang II and inhibit the degradation of bradykinin, a potent vasodilator peptide (figure 10)[219]. Nevertheless, research indicates that Ang II generation via ACE-independent pathways (serine protease chymase) may be more pronounced than ACE-dependent pathways in specific tissues and conditions. This alternative pathway remains unaffected by ACEIs, raising significant clinical implications of this medication[220]. Notably, a study showed that ACEI treatment failed to reduce Ang II levels in the interstitial fluid of the left ventricle in mice, despite effectively inhibiting ACE activity[221]. This highlights a potential limitation of ACEIs in controlling Ang II production. Consequently, ARBs were introduced[210].

As stated before, the AT_1R is primarily responsible for mediating both physiological and pathophysiological effects of Ang II, regardless of the pathway leading to its formation. Consequently, targeting AT_1R to antagonize Ang II action emerged as a logical therapeutic approach, potentially offering greater specificity than ACE inhibition[166]. The development of selective AT_1R blockers began with the synthesis of losartan. Since then, several ARBs, such as valsartan, irbesartan, candesartan, telmisartan, and olmesartan, have been developed[163]. ARB therapy increases Ang II levels by blocking the AT1R, which disrupts the negative feedback mechanism. This causes a rise in renin secretion, leading to greater production of Ang I and Ang II. The surplus Ang II can then bind to AT2 and other receptor subtypes (figure 10)[222]. The benefits of ARBs extend beyond AT_1R blockade, as they permit the activation of AT2R, which mediates additional positive effects[223].

ARBs are now widely used as first-line treatments for managing hypertension[223]. In addition to lowering blood pressure, ARB therapy has demonstrated anti-inflammatory effects and has been shown to reduce the composite risk of cardiovascular death, stroke, and progression of nephropathy, pulmonary fibrosis, peritoneal fibrosis, among other positive effects[166,195,210]. Despite the theoretical benefits of ARBs over ACEIs, ARBs have been

shown to have comparable efficacy in reducing cardiovascular outcomes (including cardiovascular mortality, myocardial infarction, and stroke) and end-stage renal failure. However, ARBs are better tolerated compared to ACEIs and have fewer side effects[210].

Aldosterone is another classical target in the pharmacological manipulation of the RAAS. Spironolactone and eplerenone, non-selective and selective MRAs respectively, are available to block aldosterone's actions[159]. These medications have improved outcomes in patients with a history of heart failure, reducing hospitalizations and mortality in those with heart failure with reduced ejection fraction[224]. However, despite their benefits, the clinical use of spironolactone and eplerenone remains limited, and they are still being studied as potential alternatives[225].

More recently the antifibrotic potential of AT_2R Agonists is being explored[226]. Additionally, ACE2 and Ang 1-7, which constitute the protective arm of the RAAS, are being investigated as potential therapeutic targets[227].

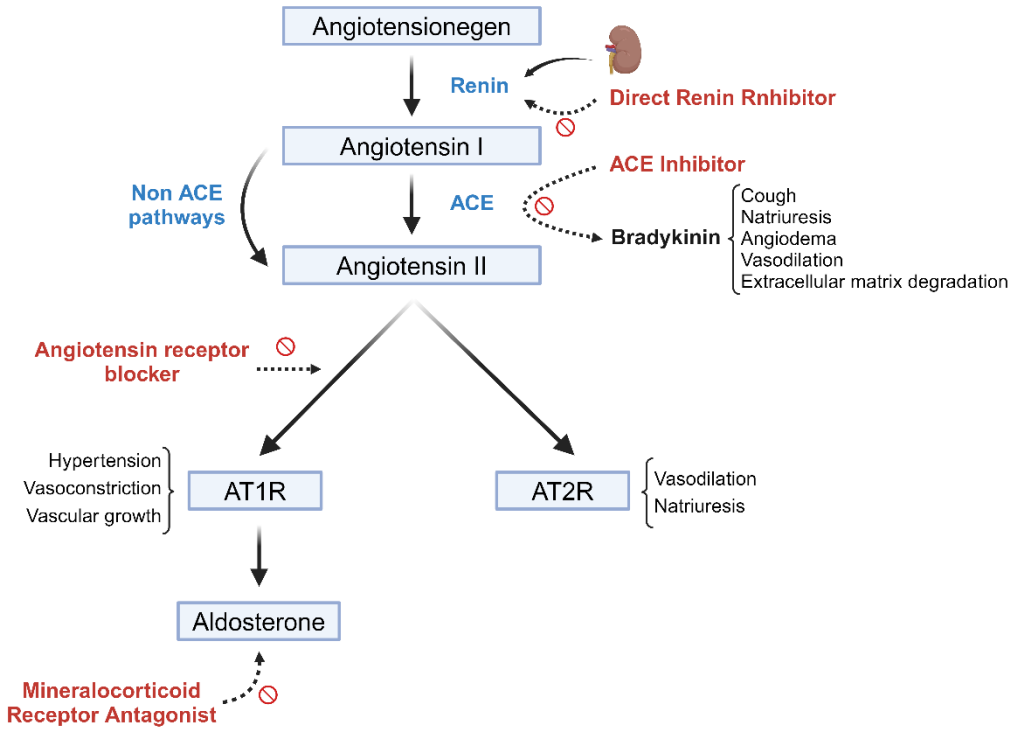

Figure 10 - Pharmacological agents targeting the renin-angiotensin-aldosterone system and their sites of action.

4.4. RAAS and the gastrointestinal tract

In addition to the systemic RAAS, there has been a growing focus on the study of the local RAAS within various organ systems. The GI tract is no exception since research has demonstrated that the GI tract expresses all the key components of the RAAS, which not only exerts a direct effect on intestinal smooth muscle function but also influences it

indirectly via the myenteric plexus cholinergic neurons. This dual modulation highlights the complexity of RAAS signaling within the GI system, affecting both local smooth muscle activity and neural communication pathways[228].

Most attention has been paid to the small intestine (figure 11a)[229]. ACE is abundant on the brush border of epithelial cells and mesenteric microvascular endothelium. ACE2 mRNA and protein are highly present in the small intestine, especially in the terminal ileum, duodenum, and colon[192]. AT_1R is located on the epithelial brush border, muscle layers, and myenteric plexus, while AT_2R is mainly in the myenteric plexus. Small vessels in the muscularis propria also express AT_1R[230]. Renin expression was detected in the human and mouse small intestine[231]. Ang II is present in crypt epithelial cells. Although angiotensinogen, Ang I, or Ang 1-7 haven't been reported in humans, angiotensinogen is found in various rat intestinal regions[229].

Research on RAAS in the colon is limited (figure 11b). AT_1R were found on crypt bases, mucosal vessel walls, lamina propria macrophages and myofibroblasts; AT2R were identified in epithelium surface, in crypts and mesenchymal cells (with less expression)[229]. Renin, ACE, and ACE2 have also been found in various parts of the colon like the surface epithelium, mucosal cells, and blood vessel walls[232,233]. Angiotensinogen mRNA has been detected in rat colon but not yet in humans[234].

Components of the RAAS are found in the stomach lining of healthy adults (Figure 11c). Renin, AT_1R, AT_2R, and ACE are present in cells like mucosal cells and blood vessels. ACE has also been seen in specific cells like mucin-secreting cells[229]. Studies show that stomach muscles respond to Ang II, indicating the presence of relevant receptors[235].

The RAAS influences gut motility and mucosal functions, such as secretion, fluid, and nutrient absorption, primarily through the activation of AT_1R and AT_2R receptors on smooth muscle cells, epithelial cells, and enteric neurons[228]. In duodenum, Ang II stimulates bicarbonate secretion via AT_1R and AT_2R[236]. In the jejunum and ileum, Ang II, in conjunction with the enteric sympathetic nervous system, modulates sodium and water absorption[237]. Ang II has also been demonstrated to inhibit glucose uptake mediated by the sodium-dependent glucose transporter in rat jejunum[238]. Brush border ACE and ACE_2 act as peptidases, aiding in peptide digestion and absorption[239].

In the colon, the RAAS appears less active than in the small intestine. Ang II enhances water and sodium reabsorption via NaCl-coupled transport[240]. Functionally, Ang II also causes muscle contraction in the colon, suggesting a role in normal bowel motility[241]. Ang II contracts circular and longitudinal smooth muscle in response to direct activation of post-junctional AT_1R and indirect activation of pre-junctional AT_1R in myenteric and submucosal neurons, inducing tachykinines and acetylcholine release[242–244]. Curiously, the human colonic smooth muscle is more sensitive to Ang II than to ACh[245], but the physiological

importance of Ang II in the GI tract is not completely understood. There is some evidence showing that Ang II is more important to sustain muscular tone than to induce phasic contractions, but further studies are needed to demonstrate it[246]. More recently, a research group[247] shown a shift from sole AT_1R activation in physiological condition to AT_1/AT_2 receptor activation during inflammation, suggesting that in pathological condition the local RAS undergoes substantial modifications[226]. Also, higher levels of Ang I and Ang II in the colon are linked to inflammation in conditions like Crohn's disease[248].

The role of the RAAS in the stomach is not well-defined. However, higher AT_1R levels in patients with Helicobacter pylori infection suggest the RAAS may play a role in stomach inflammation[249]. Animal studies suggest Ang II can worsen ulcers, suggesting a role in stomach diseases[250]. While records on the enteric RAAS system remain limited, they warrant further investigation, particularly considering its potential involvement in gastrointestinal disorders[228].

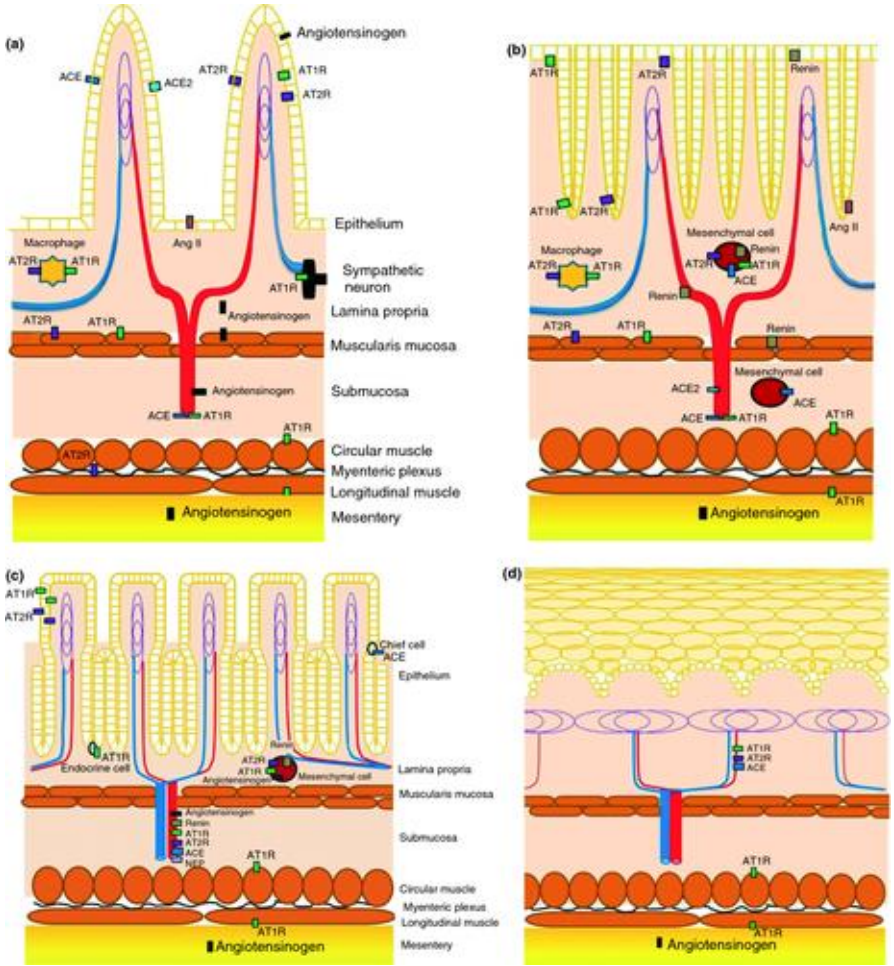

Figure 11 - Distribution of components of the RAAS in (a) small intestine, (b) colon, (c) stomach and (d) esophagus. Adapted from: Garg *et al.* 2012

4.5. RAAS and Diabetes

There is a close relationship between diabetes and the activation of the RAAS which contributes significantly to the development diabetic complications[251]. It has been shown in diabetic patients an over-activation of the classical RAAS with increased levels of circulating and tecidual ACE, leading to the increased production of Ang II and AT1R activation, contributing to increased oxidative stress and fibrosis in several organs[252,253].

The overactivation of the RAAS in diabetic patients is primarily driven by hyperglycemia, which directly and indirectly stimulates the production of Ang II[254]. Additionally, insulin resistance associated with T2diabetes is linked to increased sympathetic nervous system activity and hyperinsulinemia, both of which can enhance renin and Ang II production. Furthermore, diabetes-induced oxidative stress further activates the RAAS, leading to elevated levels of Ang II[255].

RAAS activation has been implicated in several diabetic complications in different organs such as hypertension[254], cardiomyopathy[252], nephropathy[256], and even diabetic retinopathy and neuropathy[251]. Figure 12 displays several common diabetes-related complications in the liver, kidneys, heart, and cardiovascular system.

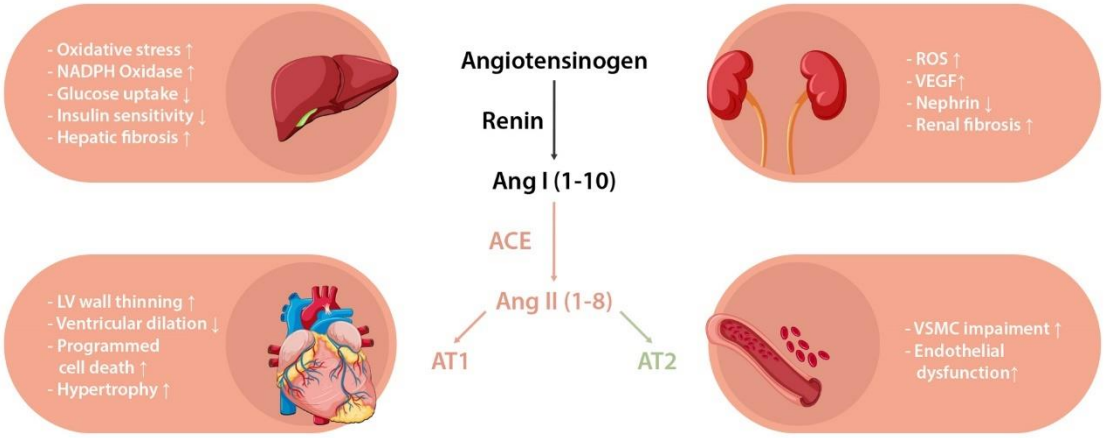

Figure 12 – Common diabetes-related complications in different organs associated with the overactivation of the local Renin-Angiotensin-System.

In the kidneys, elevated levels of Ang II contribute to cellular hypertrophy and fibrosis. This leads to structural remodeling, characterized by glomerular hypertension, increased glomerular permeability, and proteinuria[257]. Over time, these changes result in progressive loss of kidney function, culminating in diabetic nephropathy, a leading cause of end-stage renal disease[256]. In the heart, Ang II promotes cardiomyocyte hypertrophy and interstitial fibrosis[193]. These changes contribute to cardiac remodeling, which is marked by alterations in the size, shape, and function of the heart. This remodeling process can lead to diastolic dysfunction, reduced cardiac output, and eventually heart failure[252]. The pro-inflammatory

and pro-fibrotic effects of Ang II also exacerbate atherosclerosis, increasing the risk of coronary artery disease and myocardial infarction in diabetic patients[258]. Interestingly, so far, no association has been found between the RAAS and diabetes-related gastrointestinal complications.

Therapeutic strategies targeting the RAAS, particularly with ACE inhibitors and ARBs, are crucial in managing these complications and improving outcomes for diabetic patients[259]. ACE inhibitors or AT1R blockers were able to reduce the incidence of vascular complications, nephropathy and cardiovascular disease in diabetic patients[159]. In patients with T2D, inhibition of the RAAS not only provides cardiovascular and renal protection but also has beneficial effects on glucose metabolism. RAAS inhibition improves insulin sensitivity, allowing better control of glycemic values[260].

But to date, no one considered the possible therapeutic role of RAAS on diabetes impairment of intestinal motility. A recent study concluded that ACE gene polymorphism in diabetes patients is an important factor to influence gut motility and thus contributing to GI symptoms. They found that patients with T2D presented a prevalent genotype which is associated with delayed gut motility and decreased substance P level (which is decreased in patients with constipation)[261]. More recently, a paper was published that initially appears to align with the aim of this thesis. The study revealed significant diabetic-induced alterations in colonic morphology and RAAS, including increased renin receptor expression in diabetic rats. The authors suggested that this change may be influenced by the abundant microbiota present in the colon. Furthermore, enalapril treatment effectively ameliorated pathological changes in gut morphology observed in diabetic rats. This study was the first to highlight the potential of RAAS inhibition, particularly with enalapril, in alleviating intestinal complications associated with diabetes[262]. However, their focus was on gut mucosa and absorption, while this thesis emphasizes the muscular remodeling of the gut wall that may contribute to diabetic dysmotility, and the role of RAAS in this process.

5. Redox homeostasis, oxidative stress and glutathione

Redox homeostasis is the intricate balance between the generation and elimination of ROS within cells or organisms. ROS play crucial roles in cellular signaling and physiological processes[263]. However, excessive ROS production, exceeding the capacity of antioxidant defense mechanisms, leads to a state known as oxidative stress[264]. As stated before, this condition is common in diabetic patients[265] and is marked by an imbalance where ROS overwhelm antioxidants, causing damage to essential cellular components such as proteins, lipids, and DNA[263]. Oxidative stress is implicated in the pathogenesis of numerous disorders and complications[266], including gastrointestinal diseases[267]. Maintaining redox

homeostasis is critical for cellular health and overall organismal well-being[268], thus the importance of glutathione (GSH)[269].

GSH is the body's primary antioxidant, playing a crucial role in maintaining cellular health by neutralizing ROS and free radicals[264,270]. GSH is a tripeptide composed of glutamic acid, cysteine, and glycine linked by two peptide bonds. Its biosynthesis occurs in two steps, with the first step catalyzed by Glutamate–cysteine ligase, which is considered rate-limiting and dependent on the availability of cysteine. The second step is catalyzed by GSH synthetase[271]. The antioxidant role of GSH hinges on the action of glutathione peroxidase, an enzyme that plays a pivotal role in neutralizing ROS within cells. In these reactions, GSH acts as an electron donor, undergoing oxidation to form oxidized glutathione (GSSG) (figure 13)[264]. To maintain adequate levels of GSH, which is essential for ongoing antioxidant defense, GSSG is subsequently reduced back to GSH by glutathione reductase (figure 13)[272]. This reduction process consumes NADPH, a critical cofactor in cellular redox reactions, thereby completing the enzymatic cycle that sustains the cellular antioxidant capacity and preserves redox balance[273]. In physiological conditions, reduced GSH is more abundant (around 98%) than its oxidized form (GSSG)[271,274].

GSH plays a vital role in protecting the epithelial cells lining the GI tract from oxidative damage by neutralizing ROS[275]. It is abundantly present in the mucosal cells of the entire GI tract in both humans and animals, with the highest concentration found in the duodenum and stomach providing additional protection against gastric acid[276,277]. The GI tract also expresses glutathione peroxidase and reductase thar are essential to complete the redox cycle[278]. In the GI tract, GSH is not only essential for safeguarding cells from oxidative stress but also detoxifying harmful substances, supporting immune function, maintaining the mucosal barrier, influencing gut microbiota, and facilitating nutrient absorption and metabolism[276–278]. Additionally, by reducing oxidative stress and modulating pro-inflammatory cytokines, GSH helps mitigate inflammation, thereby aiding in the prevention of conditions such as inflammatory bowel disease[279]. Research has shown a direct correlation between glutathione concentration and mucosal integrity, as well as between glutathione-related enzymes and cancer incidence in various GI tract conditions, ranging from the esophagus to the rectum[267].

Measuring GSH and GSSG levels is a valuable method for assessing oxidative stress. In fact, the ratio of GSH to oxidized glutathione GSSG (GSH/GSSG) is a widely recognized marker of cellular oxidative stress[280]. Under normal physiological conditions, cells maintain a high GSH/GSSG ratio, indicating a predominantly reduced environment crucial for normal cellular functions[274]. However, during oxidative stress, the increase in ROS and free radicals leads to a significant decrease in the GSH/GSSG ratio. This can reflect a shift towards a more oxidized state (more GSSG) or a depletion of GSH. For this reason, monitoring

changes in the GSH/GSSG ratio provides insights into the redox status of cells and the extent of oxidative damage[273]. Total glutathione (the sum of GSH and GSSG) levels can also be indicative of oxidative stress. A decline in total glutathione levels often points to impaired glutathione synthesis or increased utilization due to excessive oxidative burden[281]. Assessing total glutathione provides a broader perspective on the cellular capacity to counteract oxidative stress and maintain redox homeostasis[273].

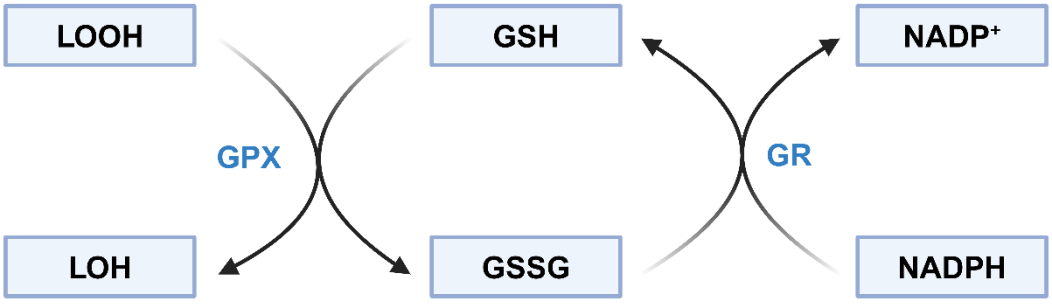

Figure 13 – The glutathione peroxidase (GPX) catalyzes the conversion reactive oxygen species such as of lipid hydroperoxides (LOOH) into lipid alcohols (LOH) using glutathione (GSH) as a reducing agent. The resulting oxidized form of GSH (GSSG) is then restored to its reduced state (GSH) by glutathione

In diabetes, both the levels of GSH and the GSH/GSSG ratio are typically affected, reflecting increased oxidative stress and impaired redox homeostasis[282]. It was already described that diabetes is associated with elevated levels of ROS in part due to the activation of RAAS[255]. In short term diabetes, increased levels of glutathione have been observed as a mechanism to combat oxidative stress[283]. However, with time the excessive ROS consume GSH due to irreversible utilization, leading to a reduction in overall GSH levels within cells[284]. The synthesis of GSH may also be compromised in diabetes due to dysregulation of the enzymes involved in its production[17]. Additionally, the availability of cysteine, a precursor for GSH synthesis, may be limited under diabetic conditions[285]. Consequently, there is a depletion of production and also a higher rate of utilization of GSH to neutralize the excessive ROS, resulting in a general depletion of GSH stores[286].

Besides the decrease in GSH seen in diabetes, there is more GSH being oxidized to GSSG, thereby lowering the GSH/GSSG ratio due to an increase in GSSG levels[287]. Furthermore, the activity of glutathione reductase, the enzyme responsible for converting GSSG back to GSH, may be impaired in diabetes due to oxidative damage to the enzyme or insufficient NADPH (the cofactor required for the reaction)[288]. This results in more accumulation of GSSG and further decrease in the GSH/GSSG ratio. This oxidative damage contributes to the complications associated with diabetes, including cardiovascular disease, nephropathy, neuropathy, and retinopathy[283,284,289].

6. Aims and Thesis Outline

This thesis is motivated by the high prevalence of gastrointestinal complications in humans with diabetes, coupled with the limited knowledge and treatment options available for these issues. Furthermore, there is a notable absence of information regarding these complications in diabetic cats and dogs. Complications related to diabetes in the intestines and colon can lead to clinical signs that significantly contribute to morbidity and may be associated with poorer outcomes in conditions such as colorectal cancer and inflammatory bowel disease. However, effective treatment options for these complications are currently lacking.

The RAAS is primarily recognized for its role in regulating blood pressure and fluid balance in the cardiovascular and renal systems. However, RAAS has also been linked to diabetes, as evidenced by the beneficial effects of ACEI or ARBs in reducing vascular complications, nephropathy, and cardiovascular disease in diabetic patients. Additionally, increased levels of tissue ACE have been observed in diabetic patients, leading to heightened formation of Ang II. Despite these findings, there remains a scarcity of information on the involvement of RAAS in diabetic gastrointestinal alterations.

Therefore, the primary objectives of this study were to address the following questions:

- How do the macro and microscopic structures of the GI tract change in both T1D and T2D rat models and which cell types are affected? Do the intestines of diabetic rats exhibit functional changes?
- Is the gastrointestinal tract altered in diabetic cats and dogs? Do diabetic pets exhibit gastrointestinal clinical signs?
- Is the expression of local RAAS enzymes (ACE, ACE2) and of the effector peptide (Ang II) altered in the gut of diabetic animals?
- Is the local activity of the glutathione system altered in the GI tract of both type 1 and type 2 rat models of diabetes?
- Can targeting the intestinal RAAS using ARBs in diabetic rat models be a therapeutic approach to prevent diabetic gastrointestinal remodeling?

So, this thesis has two main focuses. Firstly, demonstrating diabetes-related gastrointestinal complications in experimental models of both type 1 and type 2 diabetes, and experimentally testing ARBs as a preventative measure for these alterations. Secondly, conducting clinical studies in spontaneously diabetic dogs and cats, utilizing anamnesis directed towards the GI system, abdominal ultrasound for evaluating GI alterations, and *post-mortem* evaluations of diabetic pets donated for necropsy.

To explore these questions, this thesis is structured into 5 chapters:

- Chapter 1, the current chapter, reviews the existing knowledge on diabetes and its gastrointestinal complications in both humans and pets. It covers experimental models of diabetes relevant to this thesis, the Renin-Angiotensin-Aldosterone System and its relation to the gastrointestinal tract and diabetes, as well as redox homeostasis, oxidative stress, and the importance of glutathione. This chapter concludes with the aims and the outline of the thesis.

- Chapter 2 – Experimental results

2.1. The first section of this chapter focuses on the examination of the histomorphometric changes and decreased reactivity to Angiotensin II in the ileum and colon of streptozotocin-induced type 1 diabetic rats (Publication I).

2.2. Discussion of the refinement of the streptozotocin-induced model of diabetes by reducing the fasting period before induction and incorporating tramadol for analgesia, without jeopardizing the experimental results (Publication II).

2.3. Discussion of the histomorphometric gut remodeling and oxidative stress in type 2 diabetic GK rats. This section reveals intestinal and colon remodeling, changes in the neuron population of the myenteric plexus, and alterations in smooth muscle cell density. Additionally, it addresses, for the first time, local glutathione depletion and a decreased GSH/GSSG ratio in the gut of these diabetic animals (Publication III).

2.4. This section focuses on the preventive effects of losartan, an AT_1 receptor antagonist (ARB), and finerenone, a selective mineralocorticoid receptor antagonist, on GI remodeling and oxidative stress in STZ-induced diabetic rats. Additionally, it assesses ACE and ACE2 activity, as well as the ACE/ACE2 balance, in the serum and in portions of the GI tract, to characterize the circulating and local RAAS (Publication IV).

- Chapter 3 – Domestic pets results

3.1. This first section of this chapter marks the transition from laboratory animals to domestic pets. Although this publication is a systematic review rather than original research, it plays a critical role in emphasizing the value of abdominal ultrasound as a diagnostic tool for GI diseases in pets (publication V). Additionally, it offers a comprehensive overview of documented reference values for both cats and dogs, as well as essential weight categories

for dogs - key information that sets the stage for the insights presented in the following papers.

3.2. Publication VI delves into the gastrointestinal alterations in diabetic cats through ultrasound and histopathological evaluations, while also examining owners' perceptions of digestive issues. As the first study of its kind, it reveals that diabetic cats exhibit gastrointestinal clinical signs and remodeling that closely resemble those found in human diabetic patients and laboratory diabetic animals. This pioneering research offers new insights into the GI health of diabetic cats, enhancing our understanding of the condition.

3.3. This section presents preliminary results on gastrointestinal alterations in diabetic dogs, assessed through ultrasound and histopathological evaluation.

- Chapter 4 consists of a general discussion, integrating all the information from Chapters 2 and 3 and addressing the questions posed in Chapter 1. It also comprises the concluding remarks of this thesis, along with future perspectives.

- Chapter 5 includes the annex with a daily monitoring sheet created by our work group to track the streptozotocin-induced diabetic animals, as well as a QR code linking to videos of our animals spontaneously eating losartan and finerenone mixed with peanut butter.

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Chapter 2 – Experimental results

2.1. Histomorphometry Changes and Decreased Reactivity to Angiotensin II in the Ileum and Colon of Streptozotocin-Induced Diabetic Rats

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Abstract

Diabetes mellitus (DM) is a chronic progressive metabolic disorder associated with several gastrointestinal complications, affecting up to 75% of patients. Knowing that Angiotensin II (AngII) also regulates intestinal contraction, we decided to evaluate changes in ileum and colon histomorphometry and AngII reactivity in a rat model of DM. Streptozotocin (STZ, 55 mg/kg) was administered to induce DM to 24 adult male *Wistar* rats. Diabetic rats displayed all the charac-teristic signs of type 1 DM (T1DM) and fecal excretion increased about 4-fold over 14 days, while the excretion of controls remained unaltered. Compared to controls, diabetic ileum and colon presented an increase in both macroscopic (length, perimeter and weight) and microscopic pa-rameters (muscular wall thickness). Functionally, AngII-induced smooth muscle contraction was lower in diabetic rats, except in the distal colon. These differences in the contractile response to AngII may result from an imbalance between AngII type 1 (antagonized by candesartan, 10 nM) and type 2 receptors activation (antagonized by PD123319, 100nM). Taken together, these results indicate that an early and refined STZ-induced T1DM rat model already shows structural re-modelling of the gut wall and decreased contractile response to AngII , findings that may help to explain diabetic dysmotility.

Keywords: Diabetes mellitus; STZ; ileum histomorphometry; colon histomorphometry; smooth muscle contraction; Angiotensin II receptors

1. Introduction

Diabetes mellitus (DM) is a complex chronic progressive metabolic disorder, medically incurable, that can affect almost every organ system [1]. There are different animal models of DM, but streptozotocin (STZ) has been the agent of choice to chemically induce diabetes in rats and mice, causing the selective destruction of pancreatic -cells. High doses of STZ are associated with type 1 DM (T1DM) induction, while multiple low doses are usually associated with a high fat diet to cause insulin resistance, characteristic of type 2 DM (T2DM) [2–4]. In this animal model of T1DM structural, functional and biochemical alterations resemble those observed in human diabetic patients [5]. Over time, several investigators have used this model with different induction times (raising questions about animal welfare for longer protocols) in different portions of the intestine, making it harder to compare results [6–8]. For that reason, we decided to assess whether two weeks is sufficient to induce ileum and colon alterations that resemble those observed in long-lasting STZ models [9,10].

Gastrointestinal (GI) complications of DM are very important as they can be associated with significant morbidity, affecting up to 75% of patients [11]. The most common GI complications include esophageal dysmotility, gastroparesis, enteropathy and colonic disorders, such as chronic constipation and diarrhea [7,12]. Since these symptoms are not considered important causes of mortality in patients with DM they are often neglected [13]. However, it's important to recognize that they negatively influence health status and quality of life [13,14].

The pathogenesis of diabetic intestinal dysfunction seems to be multifactorial, related to the accumulation of advanced glycation end-products (AGE), injury of the enteric nervous system (ENS) or interstitial cells of Cajal, and muscular layers fibrosis [8]. Several studies also indicate that diabetic autonomic neuropathy causes damage to the ENS and changes the number and size of myenteric neurons throughout the entire GI tract in rats [15–20]. It has also been described a deficit in the intestine's cholinergic neurotransmission, since the response to exogenous acetylcholine (ACh) seems to be impaired in the ileum (30 days after STZ-induction) and colon of long-term diabetic rats (60 weeks) [21,22]. Mechanical factors can also contribute to intestinal disorders, since DM seems to cause structural remodeling that can affect histomorphometry, biomechanical properties, increase stiffness, and decrease the resting compliance and relaxation capacity of the intestinal wall [9,10,23].

The renin–angiotensin system (RAS) is mostly known for its effects in the cardiovascular and renal systems but it also has an influence in other systems, like the GI tract, which expresses all of the RAS components [24,25]. Ang II is the major effector peptide of this system, and most of its functions are mediated by the Ang II type 1 receptor (AT_1R), while

activation of the Ang II type 2 receptor (AT_2R) usually counteracts them [26,27]. In the colon, Ang II contracts circular and longitudinal smooth muscle in response to direct activation of post-junctional AT1R and indirect activation of pre-junctional AT_1R in myenteric and submucosal neurons [26–29]. Curiously, the human colonic smooth muscle is more sensitive to Angiotensin II (Ang II) than to ACh, but the physiological importance of Ang II in the GI tract is still not completely understood [25,30,31]. Interestingly, there is little information on RAS alterations in the intestine of diabetic individuals, but recently one study concluded that ACE gene polymorphism in patients with T2DM influences intestinal motility, since those patients presented a prevalent genotype that was associated to constipation [32].

Considering the above, the aim of this study was to evaluate the structural (macro and microscopic histomorphometry) and functional (smooth muscle reactivity to Ang II) impact of T1DM in the ileum and colon of a refined rat model, just two weeks after induction.

2. Results

2.1. Animal welfare and monitorization

STZ-induced rats had an initial glycemia of 99.30 ± 3.29 mg/dL that increased to 395.09 ± 13.80 mg/dL within 48 hours ($p<0.0001$, $n=23$), while control rats had an initial glycaemia of 105.63 ± 6.31 mg/dL that was roughly the same within 48 hours (111.14 ± 5.41 mg/dL; $p>0.05$, $n=8$). At d7 and d14, almost all STZ rats presented with a glycemia above 500mg/dL, while control animals presented glycemic values of 105.57 ± 4.76 mg/dL ($n=8$) at the 14th day.

The parameters documented during the daily monitorization (body weight, water/food intake and fecal excretion) are shown in Figure 1. In the control group ($n=8$), rats progressively gained weight, their weight being 7.8% ± 0.73% higher by d14 than on d0 (before fasting). Diabetic rats ($n=21$) had a consistent weight loss that was more pronounced on d2 (5% less compared to the previous day) and then maintained that weight for the remainder of the protocol (7.66 ± 1.04% lower at d14 when compared to the initial weight before fasting) (Figure 1a). Water intake was significantly higher in diabetic rats comparing to controls that maintained a constant water intake through all the experimental protocol: 37.54 ± 0.53 mL/day ($n=8$). The STZ group drank more water since d1 (48.38 ± 1.16 mL), but their water intake increased progressively throughout the protocol, reaching values 7 times higher than those of control animals at d14: 264.08 ± 12.18 mL ($n=16$) (Figure 1b). Despite the weight loss, STZ rat's food intake was significantly higher than controls after the 3rd day. Diabetic rats started the experimental protocol eating 13.25 ± 1.86 g in the first day, and

progressively increased food consumption until the last day, when the intake was 49.08 ± 2.64 g/rat (*n*=16). The control group maintained a constant food intake during the experimental time, with a mean consumption of 22.44 ± 0.38 g/day (*n*=8) (Figure 1c).

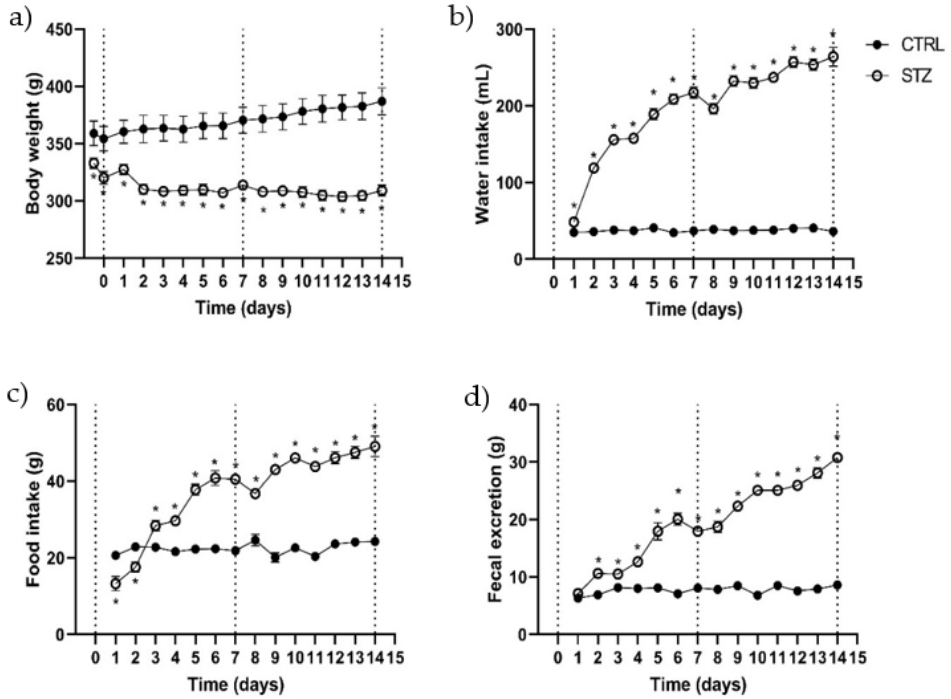

Figure 1. Evaluation during the experimental protocol (14 days) in control (CTRL, *n*=8) and streptozotocin-induced diabetic rats (STZ, *n*=16-21) of: a) body weight; b) water intake; c), food intake and d) fecal excretion. Values are mean±SEM and unpaired student's t test was used to compare the two experimental groups (CTRL and STZ). *Statistical difference, $p<0.05$.

To our knowledge, this is the first study to quantify fecal excretion in STZ-induced diabetic animals. Non-diabetic animals maintained a relatively stable fecal excretion during the entire experimental period (7.75 ± 0.18 g/day/rat, *n*=8), whereas diabetic rats gradually increased their fecal excretion, reaching values 4 times higher than those obtained in the first day (d1: 7.11 ± 0.34g/rat; d14: 30.79 ± 0.73g/rat; $p<0.0001$, *n*=16) (Figure 1d).

2.2. Ileum and colon macroscopic evaluation

Comparing to control animals, all segments of the intestines of STZ seemed enlarged. Also, upon the opening of the abdomen of STZ-induced rats it was easily perceived an extremely dilated cecum that produced a “mass effect”, pushing the intestine to the side. The colon length was significantly higher in diabetic animals compared to the control group (Figure 2a and Figure 2b: 25.75 ± 0.77cm, *n*=14 vs 19.63 ± 0.47cm, *n*=12, $p<0.05$). Since some animals were heavier than others, colon length *per* body weight was measured and the difference between the two groups was maintained (Figure 2b). The circumferential

perimeter of the intestinal portions was also measured, being significantly higher in the STZ-induced rats (*n*=11) compared to non-diabetic rats (*n*=8) both in the colon (15.45 ± 0.58mm vs 11 ± 0.46mm, *p*<0.0001, respectively) and ileum (12.55 ± 0.31mm *vs* 9.38 ± 0.32mm, *p*<0.0001, respectively) (Figure 2c). The relative weight of the whole intestine segment studied (with fecal content) was higher in STZ-induced animals than in controls (2.69 ± 0.10 g/g of body weight, *n*=21 *vs* 1.80 ± 0.05 g/g of body weight, *n*=12; *p*<0.0001, respectively). This increase was also observed at the individual intestinal segments free of fecal content (Figure 2d). Furthermore, no differences were found between STZ-induced animals and controls in the wet-to-dry ratio of all the segments studied (ileum: 5.23 ± 0.37 *vs* 5.61 ± 0.33; PC: 5.17 ± 0.24 *vs* 4.52 ± 0.20; MC: 4.84 ± 0.30 *vs* 5.16 ± 0.21; DC: 5.07 ± 0.20 *vs* 4.86 ± 0.28, respectively, *p*>0.05 for all). The 2-way ANOVA results showed an interaction between the experimental group (control or STZ) and the intestinal segments (*p*<0.0001), in accordance with our visual observation of the marked dilatation of the intestine in STZ-induced animals. The relative fecal content weight was also higher in STZ-induced animals than in controls (7.10 ± 0.15 g/g of body weight, *n*=21 *vs* 2.66 ± 0.11 g/g of body weight, *n*=12; p<0.0001). To our knowledge, this is the first time that the weight of intestinal content is reported in STZ rats.

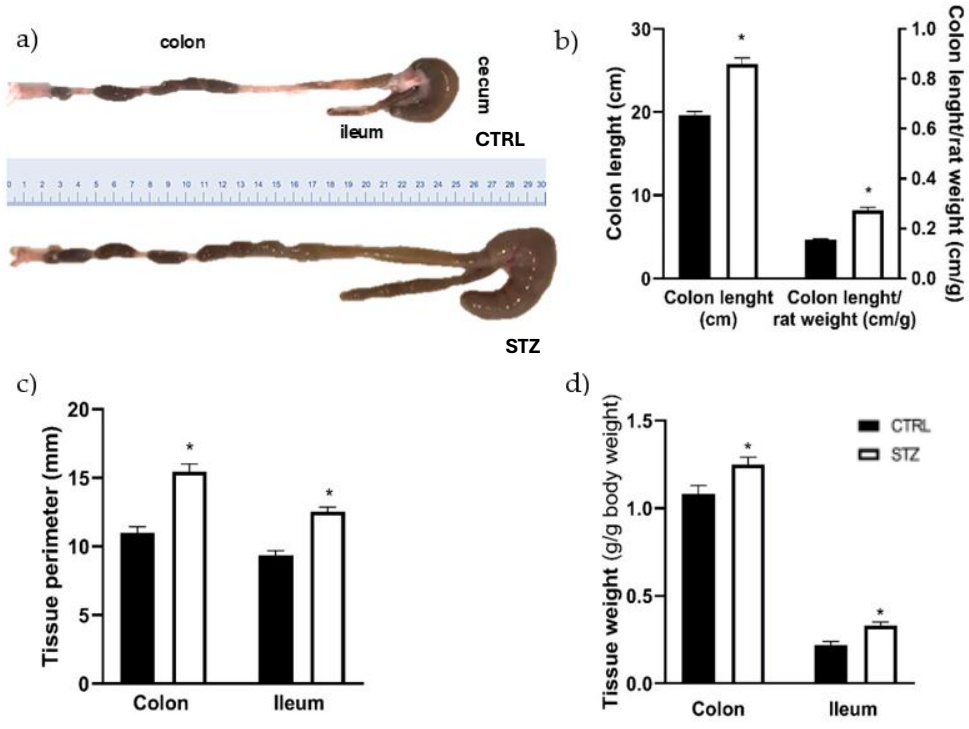

Figure 2. – Macroscopic evaluation of the ileum and colon of control (CTRL, black bars, *n*=8-12) and streptozotocin-induced diabetic rats (STZ, white bars, *n*=11-14): a) representative images of the colon length; b) quantitative analysis of colon length (left y axis) and colon length *per* rat weight (right y axis); c) tissue circumferential perimeter of the colon and ileum and d) relative weight of intestinal segments (without fecal content) expressed as g of colon or ileum/g of body weight. Values are mean±SEM and unpaired student's t test was used to compare the two experimental groups (CTRL and STZ). *Statistical difference, *p*<0.05.

2.3. Ileum and colon microscopic evaluation

The results of the histomorphometric evaluation of the intestines of STZ-induced animals (*n*=8) were concordant with the macroscopic data, showing an increase in the thickness of the intestinal wall of the ileum, PC, MC and DC compared to controls (*n*=4), as can be observed in Figure 3 and Figure 4a (ileum: 671.64 ± 74.34 µm *vs* 404.97 ± 82.04 µm; PC: 666.66 ± 32.340 µm *vs* 389.24 ± 39.03 µm; MC: 589.03 ± 17.88 µm *vs* 376.06 ± 50.62 µm; DC: 570.93 ± 27.16µm *vs* 430.42 ± 26.26µm, respectively, $p<0.01$ for all). The intestinal wall thickness increase was similar for all the intestinal segments, as 2-way ANOVA showed a non-significant association ($p=0.1681$) between experimental group and intestinal segment. Both ileum (longitudinal muscle: 81.02 ± 7.66 µm *vs* 31.18 ± 5.44 µm, circular muscle: 116.12 ± 4.59 µm *vs* 44.47 ± 10.40 µm, submucosa: 41.68 ± 1.68 µm *vs* 17.47 ± 2.13 µm, mucosa: 432.82 ± 20.59 µm *vs* 311.85 ± 24.51 µm, respectively, $p<0.01$ for all) and middle colon (longitudinal muscle: 48.93 ± 2.93 µm *vs* 29.66 ± 4.25 µm, circular muscle: 142.55 ± 8.37 µm *vs* 74.31 ± 10.9 µm, submucosa: 56.39 ± 4.09 µm *vs* 35.63 ± 6.47 µm, mucosa: 341.17 ± 13.79 µm *vs* 236.46 ± 34.58 µm, respectively, $p<0.05$ for all) presented increased thickness of all the intestinal layers assessed in STZ-induced rats compared to controls (Figure 4b). In the proximal colon, the submucosa was the only layer that presented a similar thickness between STZ-induced animals and controls (50.47 ± 7.33 µm *vs* 33.81 ± 6.00 µm, respectively, $p=0.1104$), while all the other segments were thicker in diabetic animals compared to controls (longitudinal muscle: 57.02 ± 6.90 µm *vs* 34.64 ± 4.29 µm, circular muscle: 205.2 ± 17.00 µm *vs* 90.14 ± 11.33 µm, mucosa: 353.97 ± 14.27 µm *vs* 230.64 ± 26.18 µm, respectively, $p<0.05$ for all). Distal colon only showed an increase in the muscle thickness (longitudinal muscle: 52.51 ± 2.72 µm *vs* 28.51 ± 1.67 µm, circular muscle: 150.54 ± 14.58 µm *vs* 87.21 ± 7.06 µm, $p<0.01$ for both; submucosa: 66.11 ± 7.70 µm *vs* 53.27 ± 7.54 µm and mucosa: 301.77 ± 10.00 µm *vs* 261.42 ± 16.49 µm, $p>0.05$ for both) (Figure 4B). The 2-way ANOVA showed an association between the experimental group (control *vs* STZ) and the intestinal layers thickness (longitudinal muscle, circular muscle, submucosa and mucosa) for the ileum ($p=0.0058$), PC ($p=0.0002$), MC ($p=0.0027$) but not for the DC ($p=0.1109$).

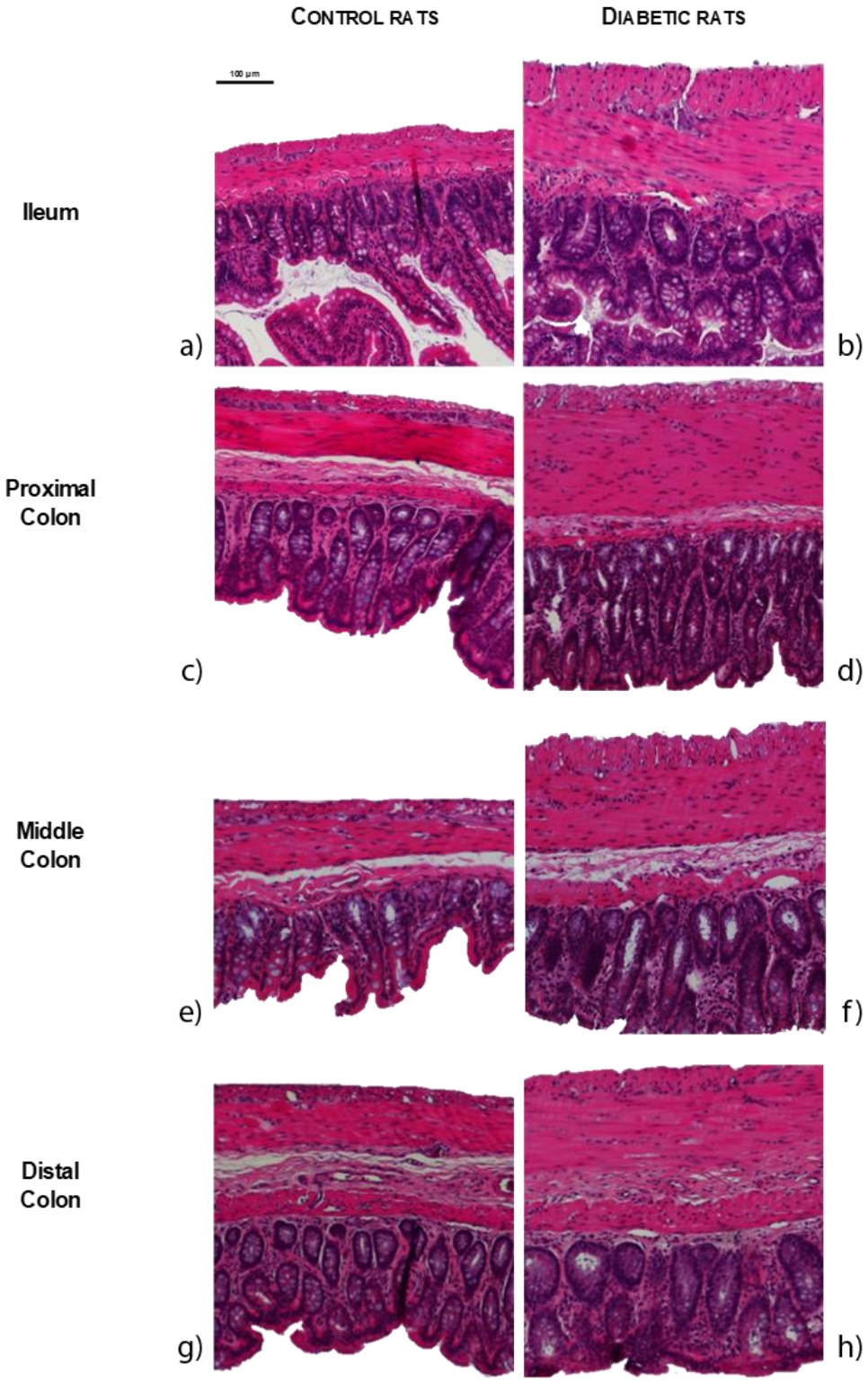

Figure 3. - Representative microscopic photographs of intestinal segments of control (CTRL, a, c, e, g) and streptozotocin-induced diabetic rats (STZ, b, d, f, h), stained with hematoxylin and eosin: ileum (a, b); proximal colon (c, d); middle colon (e, f) and distal colon (g, h). The scale bar (100 µm) is valid for all images.

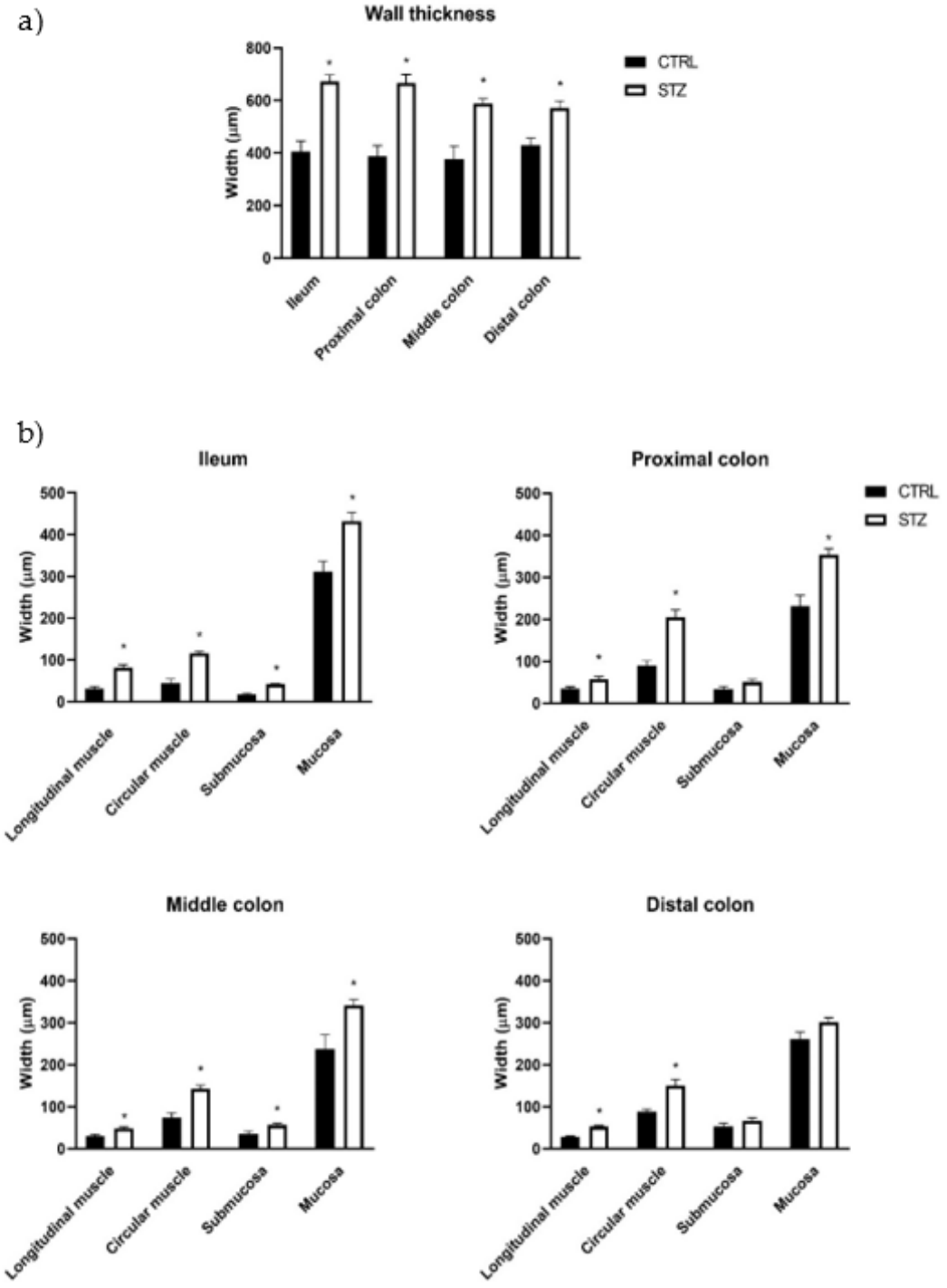

Figure 4. – Morphometric evaluation of intestinal segments (ileum, proximal colon, middle colon and distal colon) of control (CTRL, *n*=4) and streptozotocin-induced diabetic (STZ, *n*=8) rats: a) total wall thickness (µm) of each intestinal segment; b) thickness (µm) of the intestinal layers (longitudinal muscle, circular muscle, submucosa and mucosa) of each intestinal segment. Values are mean±SEM and a 2way ANOVA followed by an unpaired t test with Welch's correction was used to compare the two experimental groups (CTRL and STZ). * Statistical difference $p<0.05$ *vs* correspondent control.

2.4. Ileum and colon functional evaluation

To assess whether intestinal muscle contraction is altered in diabetic animals, ileum and colon reactivity to exogenously applied KCl, ACh and Ang II was evaluated. For the concentration-response curves to ACh (Figure 6) and Ang II (Figure 7) the results were expressed using two recognized pharmacological concepts: the maximum contractile effect (Emax, expressed in mN/g) and the concentration of agonist capable of causing 50% of the maximal contraction (EC50, expressed in uM). In all intestinal segments (ileum, PC, MC and DC) the contractile response to 125 mM KCl (Figure 5) and the ACh concentration-dependent contraction were similar in both control and STZ-induced animals (Figure 6), with comparable Emax and EC50 values, presented in Table 1.

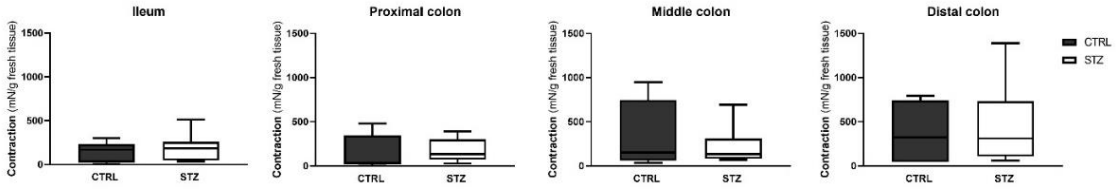

Figure 5. - Contractile response to KCl (125 mM) in the ileum, proximal colon, middle colon and distal colon of control (CTRL, *n*=6) and streptozotocin-induced diabetic rats (STZ, *n*=10). Data is expressed as mN of force *per* g of fresh tissue (mN/g). Values represent the median (95% confidence limits) and a Mann-Whitney test was used to compare the two experimental groups (CTRL and STZ). * Statistical difference $p<0.05$.

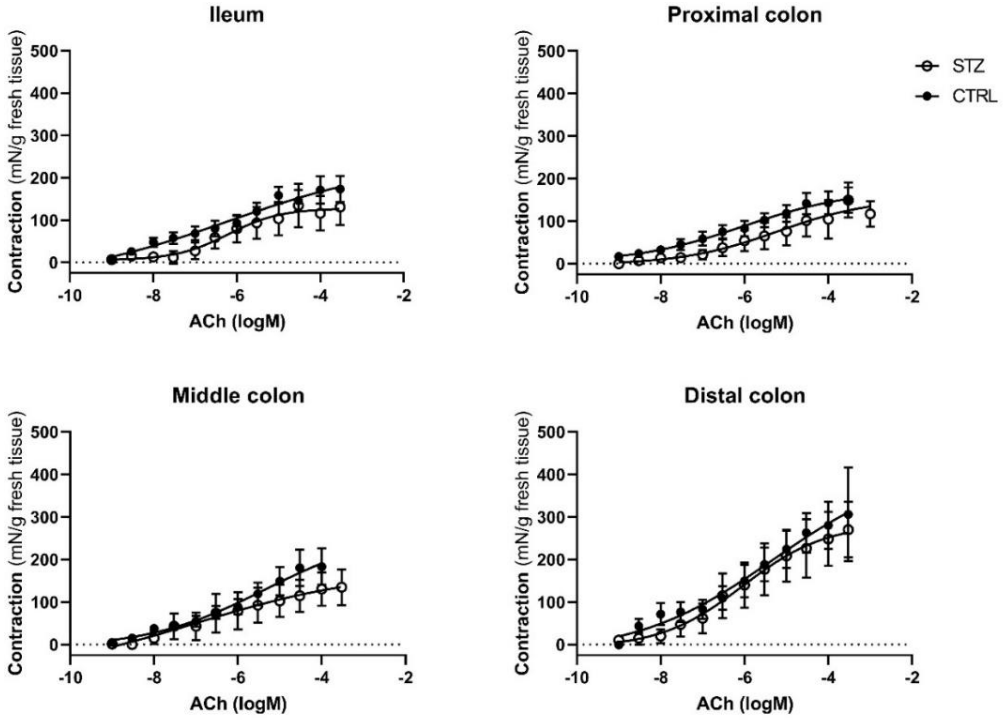

Figure 6. – Concentration-response curves to ACh in the ileum, proximal colon, middle colon and distal colon of control (CTRL, *n*=6-7) and streptozotocin-induced diabetic rats (STZ, *n*=10). Data is expressed as mN of force *per* g of fresh tissue (mN/g). Values are mean±SEM.

Table 1. – Emax (mN/g) and EC50 (μM) values of smooth muscle contraction induced by ACh application in the ileum, proximal colon, middle colon and distal colon of control (CTRL, *n*=6-7) and streptozotocin-induced diabetic rats (STZ, *n*=10).

	Ileum	Proximal colon	Middle colon	Distal colon
CONTROL				
E_{max} (mN/g)	165.9 [116.4-216.0]	141.0 [116.7-278.5]	184.7 [68.95-378.8]	313.4 [176.2-823.1]
EC_{50} (μM)	0.85 [0.32-3.53]	1.15 [0.22-14.70]	3.41 [1.1-4.8]	2.74 [0.94-7.47]
STZ				
E_{max} (mN/g)	79.06 [34.65-338.9]	158.0 [75.0-569.5]	143.6 [86.56-411.3]	271.7 [163.6-370.9]
EC_{50} (μM)	0.82 [0.27-1.87]	114.0 [8.31-3408]	18.96 [0.87-75.7]	2.94 [0.28-142.0]

For comparison between the two experimental groups (CTRL and STZ) we used a Mann-Whitney test. Values are median (95% confidence limits). * $p<0.05$ *vs* correspondent control.

Regarding reactivity to Ang II, this RAS effector peptide caused a concentration-dependent contraction in control and diabetic animals (Figure 7). The contractile response to Ang II normalized to the tissue weight was lower (but with the same EC50) in the ileum, proximal colon and middle colon of STZ-induced animals. Interestingly, the maximum response in the distal colon was similar between control and STZ-induced animals, but the EC50 of that portion of diabetic colon was significantly lower than that of controls (Table 2).

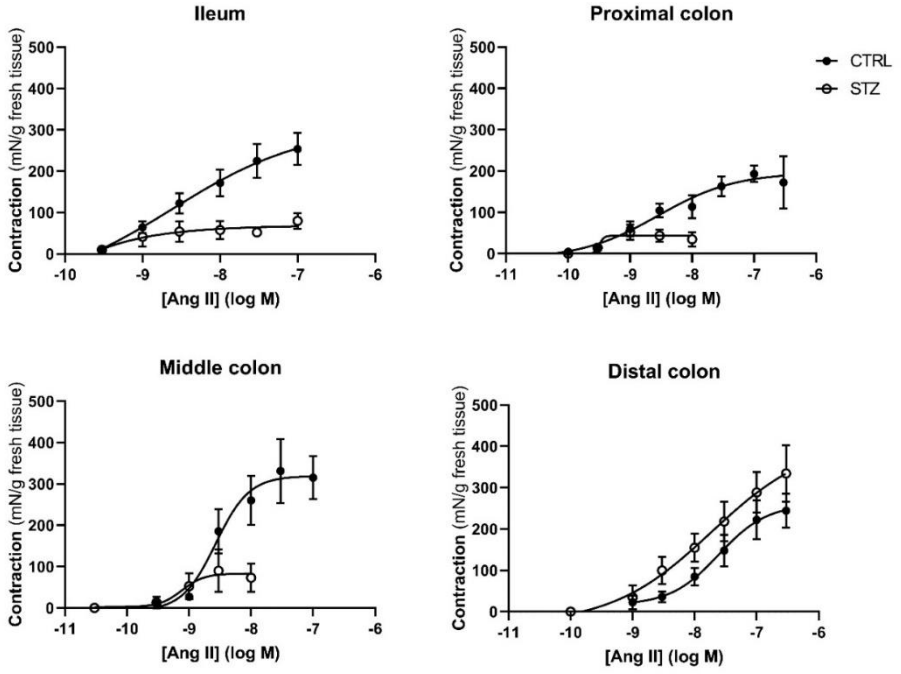

Figure 7. – Concentration-response curves to Angiotensin II in the ileum, proximal colon, middle colon and distal colon of control (CTRL, *n*=5-8) and streptozotocin-induced diabetic rats (STZ, *n*=5). Data is expressed as mN of force *per* g of fresh tissue (mN/g). Values are mean±SEM.

Table 2. - Emax (mN/g) and EC50 (μM) values of smooth muscle contraction induced by Angiotensin II application in the ileum, proximal colon, middle colon and distal colon of control (CTRL, *n*=5-8) and streptozotocin-induced diabetic rats (STZ, *n*=5).

	Ileum	**Proximal colon**	**Middle colon**	**Distal colon**
CONTROL				
E_{max} (mN/g)	305.3 [138.6- 620.5]	181.5 [136.0-297.0]	276.6 [246.4-451.1]	344.4 [222.4-433.5]
EC_{50} (μM)	8.29 [1.24-24.68]	1.10 [0.36-2.12]	3.80 [1.95-4.76]	40.50 [17.08-309.3]
STZ				
E_{max} (mN/g)	**71.20** [12.3 -100.6]*	**50.46** [15.32-78.15]*	**100.6** [22.86-163.5]*	263.5 [165.0-415.9]
EC_{50} (μM)	7.985 [0.31-8.89]	0.59 [0.35-14.93]	2.60 [0.89-7.81]	**4.17** [0.84-8.38]*

For comparison between the two experimental groups (CTRL and STZ) we used a Mann-Whitney test. Values are median (95% confidence limits). * $p<0.05$ *vs* correspondent control.

Knowing that the differences observed in the contractile response to Ang II could result from an imbalance between AT_1R and AT_2R mediated effect, we decided to further characterize the response to Ang II. The contractile response to Ang II was antagonized by candesartan (10 nM), an AT_1R antagonist, in all four intestinal segments of both control (in mN/g for all, ileum: 54.20 ± 4.50 *vs* 2.35 ± 1,60; PC: 17.37 ± 3.14 *vs* 1.07 ± 0.49; MC: 12.42 ± 2.23 *vs* 0.28 ± 0.15; DC:15.85 ± 1.32 *vs* 0.1 6± 0.08; $p<0.05$ for all) and STZ-induced rats (ileum: 35,75 ± 11,06 *vs* -0,87 ± 2,78; PC: 24.80 ± 9.45 *vs* 0.7 8± 1.19; MC :95.86 ± 29.03 *vs* 5.20 ± 6.39; DC: 288.48 ± 49.08 *vs* 5.57 ± 5.54; $p<0.05$ for all) (Figure 8a). Differently, PD123319 (AT2R antagonist, 100nM) decreased the response to Ang II in the ileum (12.43 ± 1.03 mN/g *vs* 11.02 ± 1.21 mN/g, $p<0.05$) and increased the response in all colonic segments of control animals (in mN/g for all, PC: 19.95 ± 3.34 *vs* 22.02 ± 3.45; MC: 14.99 ± 1.97 *vs* 17.48 ± 2.44; DC: 19.88 ± 2.82 *vs* 23.50 mN/g ± 2.64; $p<0.05$ for all), but was unable to modify Ang II-induced contraction in the ileum (92.58 ± 21.23 mN/g *vs* 104.24 ± 23.50 mN/g), MC (146.13 ± 18.53 mN/g *vs* 127.88 ± 21.89 mN/g) and DC of diabetic rats (236.37 ± 19.03 mN/g *vs* 248.38 ± 25.64 mN/g; $p>0.05$ for all), decreasing it in the PC (166.14 ± 20.49 *vs* 108.45 ± 19.00; $p<0.05$) (Figure 8b).

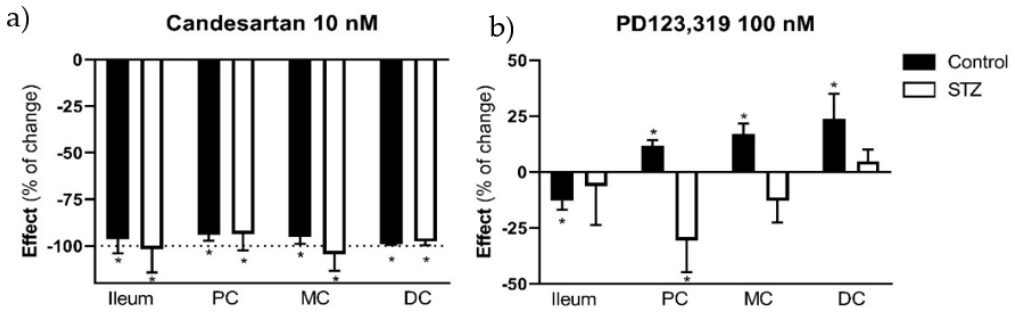

Figure 8. - Angiotensin II contractile effect (expressed as percentage of change) in the ileum, proximal colon (PC), middle colon (MC) and distal colon (DC) of control (CTRL, *n*=5-8) and streptozotocin-induced diabetic rats (STZ, *n*=4-6) in the presence of the following antagonists: a) candesartan (AT_1R antagonist, 10 nM) and b) PD123,319 (AT_2R antagonist, 100 nM). Values are mean±SEM. For statistical analysis we used a paired t test between the effect in the absence and presence of the antagonist.* $p<0.05$ *vs* the correspondent response to Angiotensin II in the absence of the antagonist.

3. Discussion

Our data show that the diabetic rat model chosen (DM chemically induced by an IP STZ injection, maintained for 14 days) presented all the typical signs of T1DM: body weight loss, polyphagia, polyuria and polydipsia [33–37]. In addition, diabetic rats gradually increased their fecal excretion whereas non-diabetic animals maintained a relatively stable fecal excretion during the entire experimental period. As pointed before, this is the first study to quantify fecal excretion in STZ-induced diabetic animals. Besides the increase in mass, the fecal pellets from the diabetic group were well formed but were larger, wider and darker than those from the control group. These findings could eventually be attributed to polyphagia and intestinal distension, differing from Cuervas-Mon and collaborators data, who described STZ-induced diabetic rats' feces as thick and amorphous, compared to those of control animals [38].

To our knowledge, this study is the first to show that the colon length and the perimeter of the ileum and colon are increased in this early DM model, and that the differences between control and STZ correlate to the different portions studied, in accordance with our visual observation of the marked dilatation of the intestine in STZ-induced animals.

Indeed, enlargement and increased length of the intestine and colon of STZ rats was already described by others, 10 and 8 weeks after DM induction, respectively [39,40]. A possibly explanation for the increase in colon and intestine length described in these studies is the remodeling of the extracellular matrix (due to increased production of collagen type 1) and AGE accumulation [40]. In this study we decided to measure only the length of the colon, as it is macroscopically difficult to distinguish ileum boundaries. Our data also shows that just 2 weeks after induction, STZ-induced rats present an increase in ileum and colon

weight. Forrest and colleagues found that dry colon weight increased significantly in diabetic animals (8 weeks after induction) compared to controls and suggested that this could be related to increased colon length, since weight per length did not differ between the two experimental groups [35]. Others observed that weight, but not length, of insulin treated diabetic rats was significantly higher compared to controls, thus contradicting Forrest and collaborators [41,42]. A possible explanation for the intestinal wall weight increase may be related to the tissue water content, which has been reported to be higher in diabetic animals [39]. However, we did not observe any difference between control and STZ-induced animals in the wet-to-dry ratio of the intestinal segments studied, results that are corroborated by other researchers [35]. For the time being, there is no clear answer as to which mechanisms are triggering the intestinal mass increase in diabetic animals, but Jervis and colleagues suggested that this enlargement could be an adaptation to polyphagia, a characteristic sign of the disease, since intestinal smooth muscle cells are plastic and adapt to functional demand, by remodeling [43]. Curiously, other causes of polyphagia such as lactation or hypothalamic lesions seem to induce similar intestinal consequences [44–46]. On the other hand, another study revealed that even when the food intake of diabetic rats was matched to that of controls, the intestinal weight of diabetic animals remained higher [9].

Our study innovatively uncovers several early histomorphometric alterations in the ileum and colon of T1DM rats and these alterations did not differ according to the different portions studied. Indeed, there are no previous histopathological data on the colon of STZ-induced rats just 2 weeks after induction, although a previous study showed similar results in the ileum 7 and 14 days after induction [9]. The same authors also studied histological characteristics of the middle colon, reporting increased intestinal wall thickness in longer STZ-induced models (4 and 8 weeks after induction) compared to controls [10].

Contrary to what happens when we look at the intestinal wall as a whole, the differences seen by layers are determined by the portion studied. This occurs since in the distal colon only the muscle layers are affected. So, the variation in thickness of the layers of the intestinal wall between diabetic and control animals becomes progressively less evident in the proximal-distal direction (from ileum to distal colon), in agreement with what was previously described by Fregonesi and collaborators [18]. This is a curious finding that reinforces the relevance of studying several intestinal segments to avoid generalizing phenomena that may occur in specific regions. Several studies indicate that increased intestinal thickness in diabetic animals may be due to: a) increased mucosa proliferation (due to higher food intake, increased expression of glucagon-like peptide 2, accumulation of AGE and/or suppression of apoptosis) and b) increased muscle layers (due to AGE mediated effects, collagen type I accumulation and/or smooth muscle cells hypertrophy) [33,40,47–51]. However, further studies are needed to understand if any of the possibilities

mentioned above explain the histomorphometric alterations observed, or if there are other mechanisms involved.

The studies conducted on ileum and colon reactivity suggest that there are no changes in the intestinal function of STZ-induced rats just two weeks after induction, since the contractile response to KCl and ACh remained unchanged in all segments studied. Previous studies using rat ileum showed a decrease in the contractile response to ACh 30 days and 6 months after STZ-induction, but this change does not seem to be related to cholinergic innervation damage or acetylcholinesterase activity modification [21,38,52]. Concerning the colon, it was not possible to find differences between the contractile response to ACh in control and STZ-induced rats, injected 30 days previously [52]. However, in a genetic model of T2DM, after a long period of disease (60 weeks) the contractile response to carbachol (an ACh mimetic) in the PC was lower than that of controls, while the response in the DC appeared to be unaffected [22]. Thus, it seems that cholinergic activity in the colon and ileum of diabetic animals may depend on several factors, such as type of diabetes, intestinal segment affected and diabetes evolution time, suggesting that main alterations in diabetic intestinal motility are probably related to changes in smooth muscle layers and non-cholinergic innervation [21,22,38].

We therefore decided, in an innovative way, to evaluate the reactivity of the ileum and colon of diabetic animals to Ang II. The results presented in the functional studies suggest a loss of contractile force in response to Ang II in the ileum, PC and MC but not in the DC of STZ-induced rats, compared to controls, probably due to the fact that the distal segments of the GI tract are the last ones to be affected by diabetic complications [18]. To our knowledge this is the first time that an altered Ang II response is reported in diabetic animals, an effect that could be associated with the structural alterations observed, loss of specific neurons (mostly in the myenteric plexus) and changes in the local tissue levels of Ang II [17,27,53]. Ang II activates both receptors in the smooth muscle cells but also presynaptic receptors in other cells crucial for colonic function, an intricate network that has been reported to be altered in the diseased colon [27,29,54]. Regarding Ang II-mediated effects, it is known that contractile responses in intestinal smooth muscle occur mainly through the activation of AT_1R, while AT_2R role according to our group and others, seem to be more important under pathological conditions [27,55,56]. Not surprisingly, we observed that the AT_1R antagonist (candesartan, 10 nM) completely abolished AngII-mediated contractile response in the ileum and all colon segments of both control and diabetic animals. However, the blockade of AT_2R with PD123319 (100 nM) was more intriguing. In the colon of control rats we observed that the AT_2R-associated counterbalance of Ang II AT_1R-mediated contractile effects was no longer present in the DC and MC of diabetic animals, and was even reversed in the PC, as we have reviewed previously [53]. Interestingly, the contractile effect of Ang II

in the ileum of control rats was decreased in the presence of PD123319. This points to a putative contractile effect mediated by the AT_2R, which although uncommon was previously described in other studies [57,58]. Even so, in the ileum this is not observed, reinforcing the idea that under pathological conditions the effect mediated by the AT_2R in the ileum and throughout the colon is loss/altered, as previously described by our group in an experimental model of colitis in rats [27,53,59].

4. Materials and Methods

4.1. Animals and housing

Since female rats seem to be less sensitive to STZ [7], forty-seven male Wistar rats with 10 to 14 weeks of age (weighing 300-400 g) were used in this study, including control (*n*=24) and diabetic (*n*=23) animals, that were distributed between the different experimental protocols. All control animals were used in the experimental procedures (since we used the same intestinal portions in different functional studies), but only eight of these rats were daily monitored in the animal house facility. Control animals were used in collaboration with other groups that collected organs such as heart, muscle and brain, in a perspective of reducing animals used in experimental research. Sample size was decided using the free software Sample Size Calculator (ClinCalc LLC®). Animals were maintained at the ICBAS-UP rodent animal house facility and the project was approved by the animal welfare body (P311/2019). This work followed the ARRIVE guidelines for reporting experiments with animals [60] (see supplementary material). Animals were maintained in a 12 hours' light/dark cycle, with controlled ventilation, temperature (20-24ºC) and relative humidity (40-60%). All animals were housed in groups of two in Sealsafe Plus GR900 Tecniplast® cages with proper bedding (Corncob ultra 12, Ultragene), with free access to autoclaved tap water (two bottles per cage) and laboratory rodent food (4 RF21, Mucedola S.r.l., Italy). Environmental enrichment such as paper tunnels and nesting material was provided in all animal cages.

4.2. Diabetes induction

On the day of DM induction (d0) animals were fasted for 4 hours (food taken from the box where the animals were housed) with free access to water. The STZ solution (S0130, Sigma-Aldrich; 55mg/ml in citrate buffer, pH 4.5) was prepared just prior to the injection, since a freshly prepared solution is considered to be more effective [4]. Diabetes was randomly induced by a single intraperitoneal injection of 55mg/kg of STZ (a concentration

that has proven successful in our group and also by other authors [61]), under the analgesic effect of tramadol (Tramal® oral suspension, 100mg tramadol/ml, Grünenthal) (20 mg/kg, PO), administered moments before [4]. The total volume of STZ solution (55 mg/kg) administered to each animal depended on its weight on the day of induction, ranging from 0.3 to 0.4mL. Rats had *ad libitum* access to water and food until the end of the protocol (day 14). Animals were considered diabetic if 48h after STZ injection their blood glucose was ≥ 250mg/dL, a situation that occurred in 23 of the 32 animal that were induced (diabetes induction success of 72%). These 23 hyperglycemic rats were included in the STZ group and used in the respective experimental protocols. Glycemia was evaluated using a FreeStyle Precision Neo (Abbott) glucometer. The blood glucose level of diabetic rats was measured by puncturing one of the tail veins at d0 (control value), d2 (to confirm or discard DM) and d7. On d14, animals were sacrificed by decapitation, using a guillotine suitable for rats (Small Guillotine, Harvard Apparatus) and blood glucose levels were obtained from blood samples collected from the abdominal aorta.

4.3. Animal monitorization and welfare evaluation

The animals included in this project were daily monitored (11:00h to 13:00h) throughout the entire protocol (d0-d14), and all information was registered in an individual evaluation table (confounders were not controlled). The evaluation started in the maintenance room, assessing the coat's appearance, piloerection, animal's posture, abdominal discomfort and changes in the breathing pattern (welfare evaluation). Then, in the observation room and with the cage open, the same parameters were observed, and the animals' hydration status was evaluated. Monitoring proceeded by weighing the animal and water/food in order to calculate daily intake. The appearance of the feces was also evaluated, and fecal pellets were weighed 48 hours after collection to assure uniform drying of all collected samples. The cages were changed every 2 days or whenever they became excessively wet due to diabetes associated polyuria.

4.4. Intestinal macroscopic evaluation

On protocol d14 control and STZ-induced rats were euthanized. The abdomen was opened, and the overall appearance of the viscera was evaluated. The abdominal aorta was identified and punctured to collect blood to measure glycemia. The ileum and colon were collected and weighed intact and after cleaning gently their content using Krebs-Henseleit solution (in mM: 118 NaCl; 4.8 KCl; 2.5 CaCl2.2H2O; 1.2 NaH2PO4.H2O; 1.2 MgSO4.7H2O; 25 NaHCO3; 0.02 Na2EDTA; 0.3 Ascorbic acid; 11 monohydrated glucose).

The longitudinal length of the colon was measured and a 1 cm portion of the ileum and middle colon was opened through the non-mesenteric border and laid flat to measure the circumferential perimeter (mm).

4.5. Intestinal microscopic evaluation

Samples (0.5 cm long) of the ileum and colon of diabetic and control animals were collected for histological examination. More precisely, the portion of the ileum was collected 3 cm proximal to the ileocecal junction; the proximal colon (PC) was collected 3 cm distal from the cecum; the distal colon (DC) 3 cm proximal to the anus and the middle colon (MC) 3 cm proximal to where the DC was collected. Each sample was opened through the anti-mesenteric border and fixed in 4% formalin. Samples were routinely processed and paraffin-embedded, cut in 3μm-thick sections and stained with hematoxylin-eosin (HE) for histological evaluation [10]. Each section was evaluated under an optical microscope (Nikon, model Eclipse E600) and photographed in 2 or 3 different representative regions with objective lens of 4x, 10x and 20x (magnification of 40x, 100x and 200x). The images were used to measure the thickness of the mucosa, submucosa, circular muscle and longitudinal muscle, always by the same person, using the free ImageJ® software. For each sample the layer thickness was measured in nine different locations and averaged. The measurements were only carried out in images where all the intestinal wall could be observed.

4.6. Intestinal functional evaluation

Four 1 cm long portions were collected from the ileum and colon of diabetic and control animals to evaluate smooth muscle contraction. The ileum was taken 2 cm proximal to the ileocecal junction; the PC 2 cm distal from cecum; DC 2 cm from anus and MC 2 cm proximal to the DC. Each sample was mounted in a vertical organ bath along its longitudinal axis, fixed to the bottom of the bath and to an isometric transducer (UGO BASILE S.R.I., Italy, Model 7004) using sewing threads. The bath was continuously aerated with carbogen (95% O2 and 5% CO2) and maintained at 37 ± 1ºC. Tissues were stretched to an initial resting tension of 1 g and mechanical responses were recorded using a PowerLab system (ADInstruments). All tissues were washed twice, every 15 minutes, and triggered with 10μM of ACh. They were then washed and allowed to stabilize for 15 minutes more before starting one of the following protocols:

- a cumulative concentration-response curve to ACh (Sigma-Aldrich, USA; 1nM to 10mM)

- a non-cumulative concentration-response curve to Ang II (Sigma-Aldrich, USA), according to the range of concentrations that was previously determined in other studies of this research group: ileum, PC and MC: 300pM to 100nM; DC: 1nM to 300nM [27]. Between each Ang II concentration tissues were washed for 1 hour (every 15 minutes), to avoid receptor desensitization.
- the response to a single concentration of Ang II (Ileum, PC and MC: 30nM, DC: 100nM) in the absence and presence of candesartan (kind gift from Dr. Fredrik Palm, Uppsala University, Sweden; 10nM, AT_1R antagonist) or PD123319 (Sigma-Aldrich, USA; 100nM, AT_2R antagonist). Tissues were incubated for 20 minutes with the antagonists before the second stimulation with Ang II.

At the end of every protocol, the contractile response to potassium chloride (KCl, 125 mM) was recorded.

Finally, each portion used in the functional study was weighed immediately after the protocol (fresh weight) and after drying for 48 h, at room temperature (dry weight). The fresh weight was used to normalize the contractile response. Fresh and dry weight were used to calculate the wet-to-dry ratio, as an index of edema, according to the following equation: $WtDr = (WetWeight - DryWeight)/DryWeight$.

4.7. Statistical analysis

The GraphPad Prism®8.1.2 software was used for statistical analysis of data. The unpaired Student's t-test was used to analyze animal monitorization and macroscopic evaluation. For comparison between 2 experimental groups (CTRL and STZ) the Student's t test was used for variables with a Gaussian distribution and the Mann-Whitney test for those with a non-Gaussian distribution. The two-way ANOVA was used to look for interaction in the data from histological evaluation and functional data. Accordingly, data was expressed as mean ± SEM for the Student's t-test and median [95% CI] for the Mann-Whitney test where "n" indicates the number of animals *per* group. In all cases, a p value of less than 0.05 was considered to denote a statistically significant difference.

5. Conclusions

The results presented in this study demonstrate that it is possible to refine a classic animal model of T1DM, improving animal welfare. In this early (two-week evolution) STZ-induced T1DM model we observed (Figure 9): 1) all the characteristic signs of T1DM (polydipsia, polyuria, polyphagia and body weight loss) and increased fecal excretion; 2) increased length, perimeter and weight in the ileum and colon; 3) increased thickness of several histological intestinal layers (less evident in DC) of the ileum and colon, and 4) decreased

Ang II-induced smooth muscle contraction (less evident in the DC) associated with altered balance between the function of Ang II receptors. These reported histomorphometric differences and altered reactivity may help to explain diabetic enteric dysmotility and will be deepen in future studies.

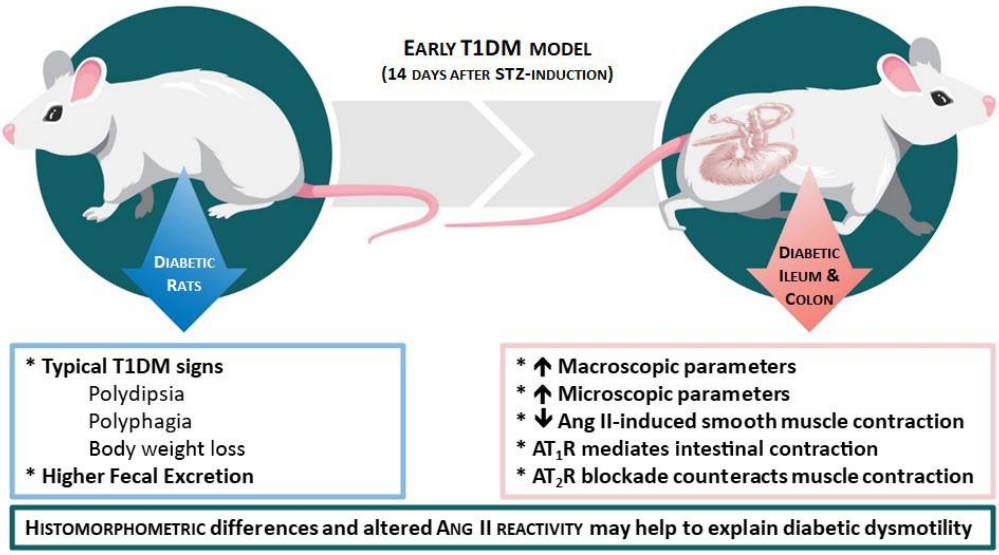

Figure 9. – Schematic representation of the major findings observed in this early T1DM model. Diabetic rats showed typical DM signs (left part of the scheme). The ileum and colon revealed an increase in both macro/microscopic parameters and a decrease in Ang II-induced smooth muscles contraction, mediated by both AT1R and AT2R activation (right part of the scheme).

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232113233/

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experiments. Animals were housed at the ICBAS-UP rodent animal facility, which is approved by the national competent authority (024159/2017 DGAV).

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2.2. Refinement of an effective streptozotocin-induced diabetic model in rats: fasting and analgesia

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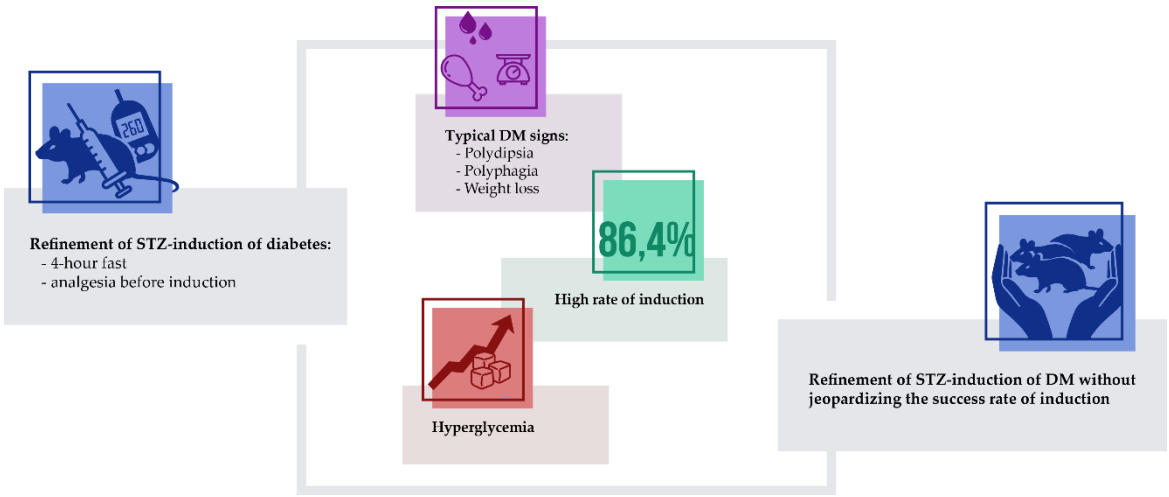
Abstract

Diabetes mellitus (DM) significantly impacts human health, justifying the need for translational studies using animal models. The widely employed Streptozotocin (STZ) induction of DM in rodents often involves a stressful prolonged fasting period without analgesia, despite the known pain associated with pancreatic inflammation. Therefore, our study aimed to assess the efficacy of STZ-induced DM under analgesia following a brief fast. DM was induced in Wistar rats (STZ, 55 mg/kg, intraperitoneal) after a 4-hour morning fast, under the analgesic effect of tramadol (20 mg/kg, *Per os*). Among the 22 animals injected with STZ 19 developed diabetes within 48 hours, yielding an 86.4% induction rate. Before fasting, baseline blood glycose levels in control and STZ-induced animals were comparable, but STZ-induced rats exhibited an increased in glycemia to 379.42±13.07mg/dL within 48 hours. By day 14 almost all STZ rats had glycemia values above 500mg/dL with ketone bodies and displayed DM classic signs: polyphagia, polydipsia, and weight loss. These findings indicate that a diurnal 4-hour fast and analgesia during STZ-induction of DM in rats can be effective and increase animal welfare without jeopardizing experimental results.



1. Introduction

Diabetes mellitus (DM) is a highly prevalent metabolic disorder primarily defined by a state of hyperglycemia(1) that has been associated with reduced life expectancy, significant morbidity, and diminished quality of life(2). Thus, it is easy to understand that there are several animal models to study this disease(3), like the chemically induced model of DM using streptozotocin (STZ)(4). STZ is a naturally occurring compound synthetized by *Streptomyces achromogenes*, with antimicrobial and chemotherapeutic properties. However, it is also known for its diabetogenic effect, caused by the selective destruction of pancreatic β-cells. In these cells glucose uptake is mediated by the low affinity glucose transporter 2 (GLUT-2). This transporter is also used by STZ to enter pancreatic β-cells, causing their destruction through two main mechanisms. Firstly, the accumulation of STZ within the cells leads to DNA alkylation, resulting in cell necrosis. Secondly, STZ triggers the production of reactive oxygen species, which accelerate the destruction of pancreatic β-cells (figure 1). The β-cells destruction impairs insulin production and secretion, ultimately leading to the development of diabetes(5). So, STZ-induced DM is widely used especially in mice and rats due to its ability to induce structural, functional and biochemical alterations that resemble those seen in human DM(4).

Since STZ enters pancreatic β-cells via GLUT-2 transporters, fasting before induction is usually recommended to avoid competition with postprandial glucose(6). However, there is a lack of consensus in the duration of the fasting in STZ-induction of DM in rats, with some investigators using up to 24 hours fasting(3,7), although the more common is 12 to 16 hours(8–10). This fasting is done mostly overnight which is the most active time for both mice and rats and when they consume about 80% of their daily food intake(11). Food restriction for so long in laboratory animals has shown to be a considerable source of stress(12) and cause other important changes. Hypoglycemia after DM induction for instance, is more pronounced in fasted animals, and it has been associated with mortality rates in mice(13). Prolonged fasting has also been reported to cause a wide range of adverse effects, like: a) loss of body weight(14); b) increase in locomotor activity in rats fasted for 18 hours(14); c) episodes of bradycardia and hypotension(15) and d) decreased body temperature (suggestive of reduced metabolic rate) in mice fasted overnight(15,16).

Furthermore, STZ induction is commonly performed without analgesia, even though pancreatic injuries, such as inflammation or cancer, are usually very painful both in animals and humans(17,18). With this in mind, we cannot assume that the pancreatic cell destruction associated with STZ administration is painless. Subsequently, we considered providing tramadol analgesia before administration of STZ. Tramadol is a central analgesic commonly used for the treatment of moderate to severe acute and chronic pain. Tramadol's analgesic effects become noticeable within 30 minutes of oral administration, offering

around 10 hours of pain relief, with an approximate half-life of 5 hours(19). However, *in vivo* studies suggest that tramadol may centrally modulate glucose levels through μ-opioid receptor agonism (20). Additionally, other authors have shown that tramadol administration is capable of lowering plasma glucose in rats with STZ-induced DM(21).

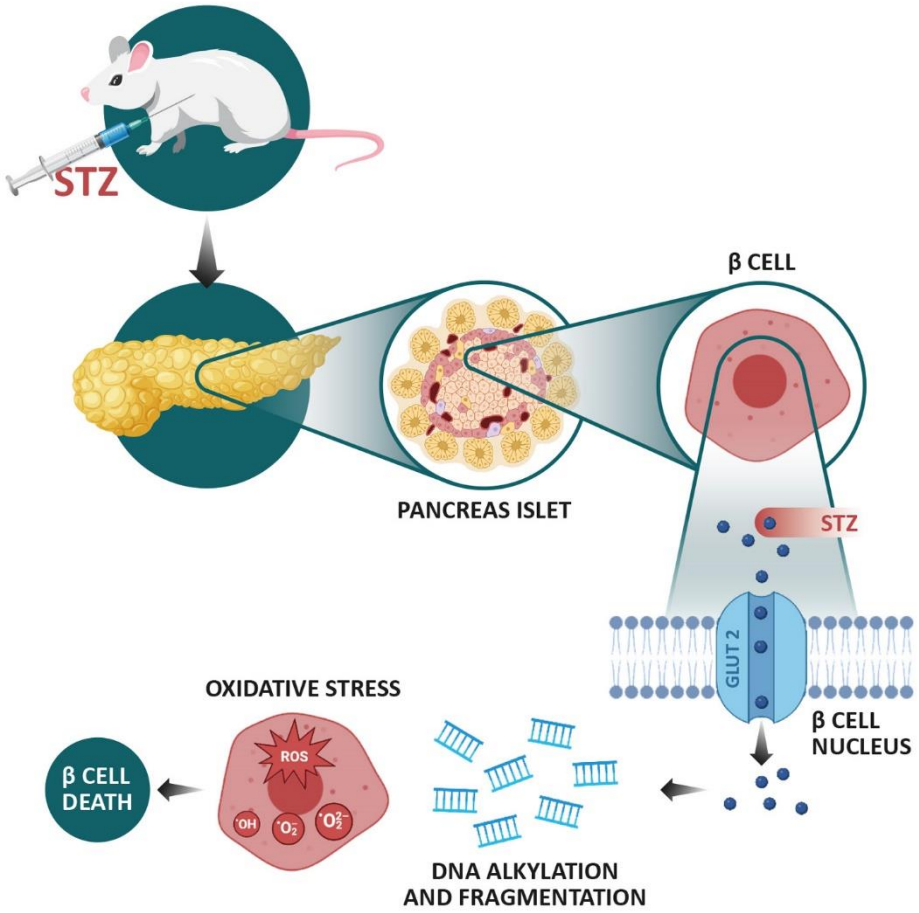


Figure 1. Mechanism of action of STZ: it enters pancreatic β-cells through GLUT-2 transporters, which are also responsible for glucose uptake. Once inside the cells, STZ induces DNA alkylation and triggers the production of reactive oxygen species, leading to the destruction of pancreatic β-cells.

The 3Rs principles (that stand for Replacement, Reduction and Refinement) were developed as a framework for the responsible use of animals in scientific research. Replacement involves finding alternative methods to animal experimentation; Reduction involves minimizing the number of animals used in experiments, without compromising the experimental goal; and Refinement involves improving the welfare of animals used in research, such as providing better housing conditions or using less invasive techniques(22). Considering the principles of the 3Rs (22), the aim of this study was to refine the STZ-induced model of DM by decreasing fasting period and providing analgesia.

2. Materials and Methods

2.1. Animals and housing

Thirty male Wistar rats with approximately 12 weeks of age (weighting 300-400 g) were used in this study. We only used males since females seem to be less sensitive to the action of STZ and usually present lower induction rates, which could represent a confounding factor(23). Sample size was calculated using the free software G*Power (α=0,05, power of 0.80 and a medium standard effect size (Cohen's d) for research on laboratory animals)(24). Animals were maintained at School of Medicine and Biomedical Sciences (ICBAS) rodent animal house facility and the project was approved by the local animal welfare body: ORBEA ICBAS-UP (P311/2019). This work followed the ARRIVE guidelines for reporting experiments with animals (25)(see supplementary material). In order to reduce the number of animals used, all the rats evaluated in this study were already assigned to another experimental protocol that studied DM-related complications(26). Additionally, control animals were also used in collaboration with other groups that collected organs such as heart, testicles, muscle, and brain (Reduction).

Animals were exposed to a 12/12 hours light-dark cycle, with controlled ventilation, temperature (20-24ºC) and relative humidity (40-60%). All animals were kept in Sealsafe Plus GR900 Tecniplast® cages with corncob bedding (Ultragene), in groups of two, with free access to autoclaved tap water (two bottles for each cage) and laboratory rodent food (4 RF21, Mucedola S.r.l., Italy). All cages were also provided with nesting paper and paper tunnels as environmental enrichment. Animal environmental conditions are described in table 1.

Table 1. Environmental conditions experienced by the animals used in this protocol.

Animals	Temperature	Humidity	Light conditions	Cages	Daily monitorization (every 24h):
30 male Wistar rats (22 STZ and 8 controls)	20-24ºC	40-60%	12h light/dark	Sealsafe Plus, 2 rats/cage	Welfare, body weight, food and water intake

2.2. Diabetes induction

On the day of DM induction (day 0), animals randomly chosen to be induced were fasted for 4 hours during the morning, with free access to autoclaved tap water. The STZ solution (55 mg/ml in citrate buffer pH 4.5, Sigma-Aldrich) was prepared just prior to the injection, since a freshly prepared solution is considered to be more effective(5). Type 1 DM (T1DM) was induced by a single intraperitoneal injection of 55 mg/kg of STZ(23), under the analgesic effect of tramadol (20 mg/kg, *Per os*), administered moments before. Since the destruction of pancreatic β-cells occurs mainly during the first 8 hours after STZ administration(5), a single oral dose of tramadol was administered. Rats maintained *ad libitum* access to water and food through the remaining protocol. Animals were considered diabetic if 48h after STZ injection their blood glucose was ≥250mg/dL(27). Glycemia was evaluated using a FreeStyle Precision Neo glucometer (small sample size > 0.6µL blood) and compatible individually wrapped test strips. The blood glucose level of diabetic rats was measured by puncturing one of the tail veins at day 0 before any procedure (control value) and day 2 (to confirm or discard DM). On day 14, glycemia was obtained after the animal's sacrifice using isoflurane overdose, followed by decapitation as a confirmatory method, collecting blood from the abdominal aorta. The protocol was carried out in two different time periods, resulting in a total of 22 animals that were used to assess the success of STZ- DM induction: 10 rats were used in the first period, while 12 animals were used in the second period. Different animals of similar age and body weight (*n*=8), that did not undergo any of these procedures, were used as controls.

Figure 2. Protocol of STZ-induced DM induction, with a 4-hour morning fast and tramadol analgesia.

2.3. Animal monitorization and welfare evaluation

All animals included in this project were daily monitored (11:00h to 13:00h) throughout the entire protocol. Information regarding their physical and behavioral status was registered in individual evaluation tables. In the maintenance room the team evaluated the animals' coat appearance, piloerection, posture before and after a brief stimulus, any abdominal

discomfort, and changes in the breathing pattern. The Grimace scale was used to evaluate pain signs(28). Afterwards, in the observation room with the cage open beneath the flow chamber, the same parameters were monitored, plus the hydration status of the animals, that was also evaluated. Then all animals, food and water were weighted, in order to calculate body weight variations and the daily intake of food and water.

After monitoring the animals, the cages were randomly assigned to different positions on the rack to reduce potential bias that should be taken into consideration in protocols where T1DM is the outcome(29). According to some authors, being housed on the top level can be a cause of stress(29), so randomizing the positioning of the cages varies the animals' exposure to light, sound, vibration, olfactory cues and visual stimuli, reducing potential bias(30).

2.4. Statistical analysis

The GraphPad Prism© 8.1.2 software was used for statistical analysis of data. The unpaired Student's t test was used for comparison between 2 experimental groups (CTRL and STZ). In order to evaluate glycemia changes within each group at different time points (Day 0, Day 2, and Day 14) repeated measures by ANOVA was used. Data was expressed as mean ± standard error of the mean (SEM) while "*n*" refers to the number of experimental animals *per* group. In all cases, a "*p* value" of less than 0.05 was considered to denote a statistically significant difference.

3. Results

The first induction of 10 rats resulted in 8 diabetic animals, while the second induction of 12 rats in the same conditions resulted in 11 diabetics. In summary, from 22 animals that were injected with STZ 19 became diabetic, representing an average rate of induction of 86,4%. None of the animals showed any signs of hypoglycemia associated with STZ injection (which usually occurs 2 to 8 hours after induction(5)) and there was no mortality.

Before fasting, basal glycemia of control and STZ-induced rats was similar (105.63 ± 6.31 mg/dL *vs* 102.21 ± 3.48 mg/dL, respectively, $n=19$ $p>0.05$). STZ-induced rats glycemia increased to 379.42 ± 13.07 mg/dL within 48 hours ($p<0.0001$, $n=19$), while control rats glycaemia was roughly the same (111.14 ± 5.41 mg/dL; $p>0.05$, $n=8$). After 14 days, almost all STZ rats presented blood glucose levels above 500 mg/dL and ketone bodies, while control animals presented glycemic values of 105.57 ± 4.76 mg/dL. The results are summarized in table 2.

Table 2. Glycemia values of control (n=8) and STZ-induced rats (n=19).

Glycemia (mg/dL) / Group	Day 0	Day 2	Day 14
STZ	102.21 ± 3.48*	379.42 ± 13.07*	>500
Control	105.63 ± 6.31	111.14 ± 5.41	105.57 ± 4.76

*- statistical difference ($p<0.05$)

The parameters documented during the daily monitorization (body weight, water and food intake) are shown in Figure 3. In the control group, rats progressively gained weight, with values reaching 7.8% ± 0.73% higher on d14 than on d0 (prior to fasting). Conversely, the fasted rats experienced a weight loss of about 1.5% on the day of induction. Diabetic rats displayed A consistent weight loss, which was more pronounced on d2 (less 5.5% compared to the previous day) and then maintained a stable lower weight. After 14 days, their weight was 6.67 ± 1.59% lower compared to the initial weight (d0, prior to fasting) (figure 3a).

Food intake was significantly higher in STZ rats compared to controls (figure 3b). Diabetic rats started the experimental protocol by consuming 12.00 ± 2.39 g on the first day, with food intake progressively increasing until the final day, when they consumed 46.13 ± 2.85 g (an average of 30.30 ± 1.40 g/rat/day). The control group maintained a constant food intake throughout the entire protocol, with a mean consumption of 22.44 ± 0.38 g/rat/day.

The STZ group exhibited significantly higher water intake (197.57 ± 16.12mL/day/rat) compared to the control group, which maintained a constant water intake throughout the entire experimental protocol: 37.54 ± 0.53 mL/day (Figure 3c). Water intake among diabetic rats increased progressively, reaching values 7 times higher than those of the control animals by d14: 258.88 ± 17.68 mL *vs* 36.00 ± 1.38 mL.

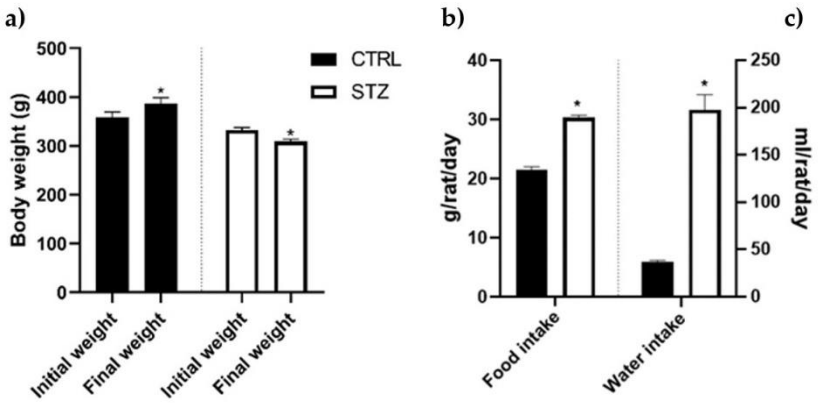


Figure 3. Parameters documented during the daily monitoring of rats: body weight difference between d0 (initial weight) and d14 (final weight) (a) and average daily food (b) and water intake (c) during the 14 days of the protocol, in both streptozotocin-induced diabetic rats (STZ) and control (CTRL) rats. *Statistical difference ($p<0.05$).

Throughout the duration of the experiment, it was noted that the diabetic animals exhibited no discernible symptoms of pain, so no additional analgesics were administered to them. However, towards the end of the 15-day protocol, it was observed that some animals displayed mild piloerection, a typical sign of discomfort. However, it was an isolated response that did not seem to affect the animals' overall health or wellbeing.

4. Discussion

These results shows that it is possible to successfully induce T1DM with a single IP injection of STZ after a fasting period of diurnal 4 hours, under the analgesic effect of tramadol. According to the literature, prior to STZ induction rats should be fasted up to 16 hours, in order to minimize competition between glucose and STZ intake by pancreatic β-cells(6). However, other authors have shown the same diabetogenic effect of STZ between fasted and fed mice(31). So, considering animal welfare, we decided to test a 4-hour fast prior to STZ administration. Food was removed earlier in the morning (07:00) to start the induction at 11:00, respecting the animal's natural circadian feeding rhythm. As so, food was available overnight (when rats are more active) and was removed in the morning, when food consumption is lower(11), reducing not only the body weight loss, but also fasting-associated stress (32). Using this refined protocol we were able to achieve an average rate of induction of 86,4%, a success rate > 80% in accordance with what's expected in DM induction (23), and higher when compared with other studies that use similar STZ dosage in male Wistar rats (33,34).

Control animals maintained normoglycemic values over time, whereas a significant increase in the glycemic values of STZ rats was observed 48 hours after induction, presenting blood glucose levels above 500 mg/dL and ketone bodies by d14. The observed hyperglycemia was due to STZ-induced toxicity towards pancreatic β-cells, that resulted in insulin deficiency. The reduced insulin secretion impaired cell glucose uptake from the blood, causing hyperglycemia. Additionally, cell glucose deficiency induces lipolysis, proteolysis, glycogenolysis and neo-glucogenesis, leading to even higher blood glucose levels(2). The long-lasting hyper-glycemia in STZ-induced rats is usually observed when induction is carried out with a single dose of STZ, resulting in blood glucose values four times higher than those of non-diabetic controls(34). Since glycemia measurements were performed both prior to any procedure and then again 48 hours after, we are confident that the tramadol administration on d0 didn't introduce any potential confounding effects on the anticipated hyperglycemia, which STZ-induced rats consistently maintained throughout the entire protocol.

Throughout this study diabetic animals exhibited all characteristic signs of diabetes: polyuria, polydipsia, polyphagia and weight loss (35). These signs have been observed in all animal models of DM, as a result of reduced insulin secretion that impairs cell uptake of glucose from the blood, ultimately leading to reduced body weight(36). The lack of glucose in the cells also creates a state of starvation that causes polyphagia as a compensatory response, without an expected increase in body weight(35). Moreover, hyperglycemia causes glycosuria with consequent osmotic diuresis, which explains polyuria and polydipsia(38). In this study, the STZ group demonstrated a significant increase in water and food intake compared to the control group, which is consistent with the findings of other researchers(39). It can be inferred from these data that rat STZ-induced DM is a highly reproducible animal model, that mimics human DM symptoms and can be used as a reliable and valid model for future investigations of diabetes and its complications.

The 3Rs principles are widely accepted and have been adopted by many countries and institutions around the world(22), and have been very important to reduce the number of animals used in research and led to significant advances in animal welfare. In this study we aimed to refine the well-known protocol of STZ-DM induction, while actively reducing the number of animals used in research, by using rats that were already assigned to another experimental protocol and by sharing organs with other research groups. The findings of this study reveal that simple modifications of the traditional STZ-induction method of T1DM in rats can provide significant benefits for the animals without increasing costs, time or requiring additional training of the professionals involved in these experimental procedures. As outlined in this paper, there are three key parameters that contribute to the refinement of the STZ-induction of T1DM with a single IP injection, while maintaining a high success rate of induction. Firstly, reducing the fasting period to just 4 hours can decrease stress and body weight loss that is commonly associated with prolonged fasts. This modification is crucial for ensuring the welfare of the animals and for obtaining accurate and reliable results from the experiments. Secondly, implementing the fasting period at a time of day when it has less impact on animal physiology and behavior(32) can further contribute to the refinement of the induction process. This may involve taking into account the natural circadian rhythm of the animals and adjusting the timing of the fast accordingly. Finally, providing analgesia with tramadol before induction reduces pain and discomfort associated with this procedure, improving not only the welfare of the animals, but also increasing the accuracy and reliability of the experimental results by minimizing the confounding effects of pain and stress on the animals' physiology.

In conclusion, our data shows that simple modifications in the traditional STZ- induction of DM protocol in rats offer a simple, cost-effective way to improve the animals’ welfare without jeopardizing the experimental outcome. These refinements should be considered by

researchers and animal care professionals in order to promote ethical and humane practices in animal research.

Author Contributions: Conceptualization: M.M. and M.D.A.; methodology: M.E.M., D.M.P., M.F.D., M.M. and M.D.A.; data analysis: M.E.M., M.F.D., M.M. and M.D.A.; investigation: M.E.M., M.F.D., M.M. and M.D.A.; writing—original draft preparation, M.E.M.; writing—review and editing: M.F.D., M.M. and M.D.A.; supervision: M.M. and M.D.A.; project administration: M.M and M.D.A.; funding acquisition: M.M. and M.D.A.. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal procedures were performed according to the Portuguese DL n° 113/2013 and European Guidelines for humane and responsible animal care (European Directive 2010/63). All protocols were approved by ICBAS-UP animal welfare body (P311/2019) in accordance with the ARRIVE guidelines for reporting experiments. Animals were housed at the ICBAS-UP rodent animal facility, which is approved by the national competent authority (024159/2017 DGAV).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is available upon request.

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Conflicts of Interest: The authors declare no conflict of interest

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2.3. Oxidative stress and histomorphometric remodeling: two key intestinal features of type 2 diabetes in GK rats

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Abstract

Gastrointestinal complications of Diabetes mellitus (DM) are often overlooked, despite affecting up to 75% of patients. This study explores local glutathione levels and morphometric changes in the gut of Goto-Kakizaki (GK) rats, a type 2 DM animal model. Compared to Wistar rats, segments of the intestine, cecum and colon were collected for histopathological analysis and glutathione quantification. A significant increase in the total thickness of the intestinal wall of GK rats was observed, particularly in the duodenum (1089.02±39.19 vs. 864.19±37.17µm), ileum (726.29±24.75 vs. 498.76±16.86 µm), cecum (642.24±34.15 vs. 500.97±28.81µm) and distal colon (1211.81±51.32 vs. 831.71±53.2 µm). Additionally, diabetic rats exhibited thickening of the muscular layers in all segments except for the duodenum which was the only portion where smooth muscle cell number was not reduced. Moreover, myenteric neuronal density was lower in GK rats, suggesting neurological loss. Total glutathione levels were lower in all intestinal segments of diabetic rats (except duodenum) and the reduced/oxidized glutathione ratio (GSH/GSSG) was significantly decreased in GK rats, indicating increased oxidative stress. These findings strongly indicate that GK rats undergo significant intestinal remodeling, notable shifts in neuronal populations, and heightened oxidative stress—factors that likely contribute to the functional gastrointestinal alterations seen in diabetic patients.

Keywords: Diabetes mellitus; GK rats; gut remodeling; oxidative stress

1. Introduction

Diabetes is a highly prevalent metabolic disorder characterized by a state of hyperglycemia [1]. The most recent data from the International Diabetes Federation indicates that diabetes affected 537 million people worldwide in 2021, a number that is expected to grow to 643 million by 2030 [2]. Besides the substantial economic impact of the disease, the importance of diabetes is also related to significant mortality and morbidity rates, being considered a major public health problem [3–5]. There are two main forms of diabetes: type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is caused by an absolute insulin deficiency while T2D is a combination of insulin resistance in target organs and relative deficiency caused by dysfunctional pancreatic β-cells [6]. T2D is far more prevalent, accounting for 90 to 95% of all cases [7, 8]. Around 80% of adult T2D patients are considered overweight or obese. However, 10-15% are not obese and those present higher hypoglycemic events and mortality rates [9]. Given the significant importance and wide prevalence of diabetes, it becomes evident that numerous animal models are employed in the study of diabetes - related complications [10]. The Goto–Kakizaki (GK) rat is a non-obese animal model of T2D that was developed by Goto, Kakizaki and Ma-saki in 1975 [11, 12]. This model was obtained by selective reproduction of non-diabetic Wistar rats with slight glucose intolerance. Consequently, the rats from posterior generations spontaneously developed T2D without becoming obese [13, 14]. GK rats exhibit reduced pancreatic β-cell number and function, moderate hyperglycemia, glucose in-tolerance and peripheral insulin resistance [15].

Diabetes frequently courses with gastrointestinal (GI) complications that are associated with significant morbidity, affecting up to 75% of patients. Currently, it is unclear whether the prevalence differs between T1D and T2D [16]. In the small intestine and colon, diabetes - related complications usually result in symptoms like chronic constipation, diarrhea, and fecal incontinence that may result in potential complications such as megacolon, pseudo-obstruction, stercoral ulcer, or perforation [17, 18]. In addition, diabetes seems to worsen clinical conditions such as colorectal cancer and inflammatory bowel disease [19]. Although highly prevalent, these symptoms are often overlooked, as they do not significantly contribute to mortality in diabetic patients. However, it is crucial to acknowledge that they negatively impact health status and quality of life, making them a significant source of morbidity [20]. The relationship between diabetes and the pathogenesis of the described gut disorders is not completely understood and seems to be multifactorial [21]. Mechanical factors contribute to intestinal disorders, since it has been reviewed that diabetes seems to cause structural remodeling that can affect histomorphometry and biomechanical properties, increase stiffness, and decrease the resting compliance and relaxation capacity

of the intestinal wall [21]. A previous study of our group has also shown significant histomorphometry changes and evidenced lower reactivity to angiotensin II of the ileum and colon of T1D-induced rats [22]. Also, Zhao et al. demonstrated the existence of remodeling in the esophagus and stomach of GK rats [23] while Pereira et al. showed alterations of the small intestine in the same animal model [24]. So far, only one study showed colon remodeling in a T2D model, associating it with the formation of advanced glycation end products [25].

In diabetes various pathways contribute to tissue damage, but a common hallmark is heightened oxidative stress, characterized by elevated levels of reactive oxygen species (ROS) [26]. Moreover, chronic hyperglycemia is linked to decreased cellular levels of glutathione (GSH) [27]. GSH is the most powerful intercellular antioxidant in the organism, undergoing oxidation to GSSG (glutathione disulphide or oxidized glutathione) after contact with electrophiles. These reactions can be catalyzed by GSH-peroxidase. The GSSG can subsequently be regenerated back to GSH by GSH-reductase, using NADPH as a cofactor, or it is excluded from the cell through membrane transporters (e.g. multidrug resistance-associated proteins, MRPs). Maintaining an optimal ratio of GSH to GSSG within the cell is crucial for survival and a decrease in this ratio may be used as a marker of oxidative stress [28]. Oxidative stress and ROS formation have already been described as markedly increased by uncontrolled hyperglycemia [29]. Also, a decrease in GSH was already described in the liver [30], erythrocytes [31] and colon [32] of long-term diabetic patients. But so far there is no data regarding GSH local levels in diabetic small intestine.

Curiously, most researchers studying diabetes -related complications in the GI tract use animal models of T1D, even though T2D is the most common form [21]. Considering this and that diabetic patients commonly present GI complications, we innovatively aimed to characterize the entire gut histomorphometry and the local glutathione system in an animal model of T2D. Examining the entire gut—from the duodenum to the distal colon—in the same animals allows for a direct comparison between segments, providing a clearer understanding of how diabetes uniquely affects each part of the gastrointestinal tract. Additionally, local glutathione levels offer a more precise picture than systemic levels because they provide insights into the specific redox environment and oxidative stress within a targeted tissue or organ—like the gut in this case. Systemic GSH levels represent an overall average throughout the body, which can mask localized changes or stresses. In contrast, studying local GSH concentrations allows us to understand how oxidative balance is maintained or disrupted in a specific region, which is particularly relevant for organs impacted by T2D, where localized oxidative stress can contribute to disease progression. To achieve this goal, we took samples of GK rats' duodenum, middle jejunum, distal ileum, cecum, proximal and distal colon and measured the indi vidual layers of the intestinal wall,

analyzed smooth muscle cells and myenteric neurons, and quantified GSH and GSSG levels.

2. Results

2.1. Animal monitorization and insulin tolerance test

GK rats presented elevated fasted glucose concentrations compared to controls (237.88 ± 81.05mg/dL vs 100 ± 1.73mg/dL, respectively, $p<0.05$) (time 0, figure 1). After a 6-hour fasting and insulin administration, the glycemia of the GK group increased during the first 30 minutes and then decreased, reaching the initial glycemic quantification at the end of the insulin tolerance teste (ITT, time 120 minutes, figure 1). In the control group, after insulin injection a slight decline in blood glucose values was observed. Compared to the control (CTRL) group, GK group blood glucose concentration was higher in all time points ($p<0.0001$, figure 1).

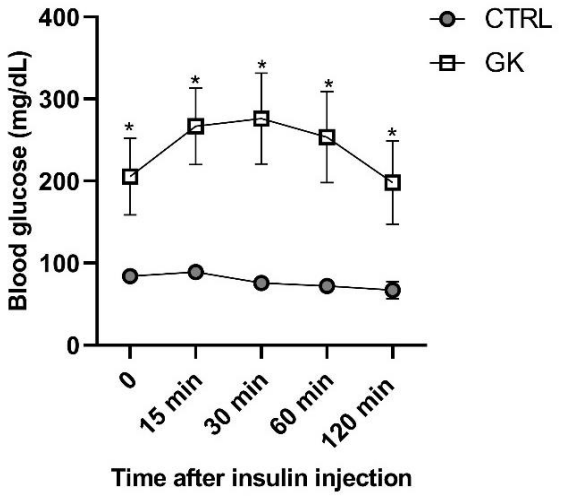

Figure 1. Blood glucose concentrations of control (CTRL, n=5) and GK animals (n=6) measured before (time 0) and during the insulin tolerance test— ITT. Values are presented as mean ± SEM, and a paired Student's t-test was used to compare the two experimental groups (CTRL and GK). *Statistical difference, $p<0.05$.

In the beginning of the protocol, the weight of the GK group was in average 329.17 ± 7.4g, increasing 2 weeks later to 340.17 ± 6.95g, representing an average of weight gain of 3.24 ± 0.62%. The controls rats weighed 402.20 ± 9.56g in the beginning of the protocol and 417.40 ± 8.81 at the end, representing an average weight gain of 3.65 ± 0.81% (figure 2a and 2b). So, the initial and final weights of GK rats were both lower compared to controls ($p< 0.0001$) (figure 2a), but the % of weight gain during the experimental period was roughly the same in the 2 groups ($p>0.05$) (figure 2b). Despite maintaining the same weight gain as controls, the food intake of GK rats (28.95 ± 1.40 mg/day/rat) was significantly higher than that of controls (21.50 ± 0.50 mg/day/rat) (figure 2c). Regarding water intake, it was

significantly higher in diabetic rats comparing to controls (figure 2d). The GK group drank 64.38 ± 5.63 mL/day/rat (n=6), which was more than double compared to control animals (30.30 ± 0.40 mL/day/rat, n=5).

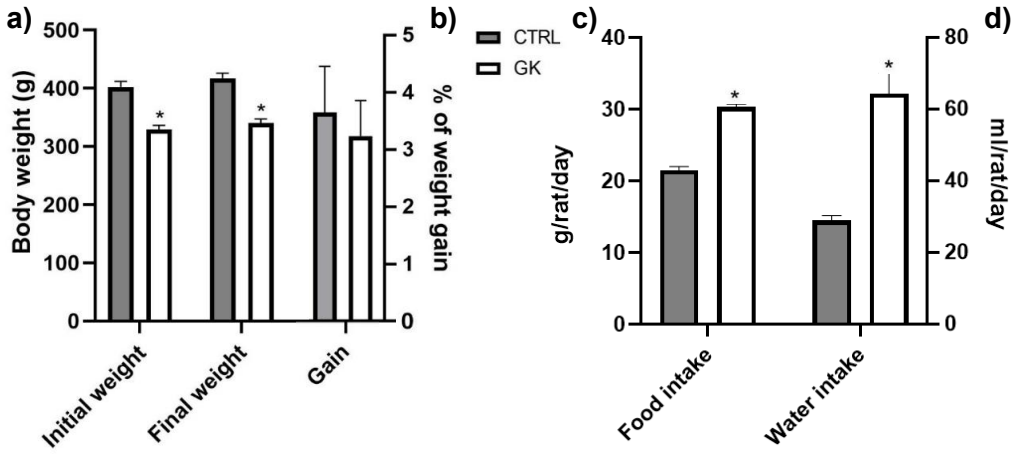

Figure 2 - Evaluation during the experimental protocol of control (CTRL, n=5) and GK diabetic rats (GK, n=6) of: (a) body weight; (b) body weight gain; (c) food intake and (d) water intake. Values are presented as mean ± SEM and unpaired student's t test was used to compare the two experimental groups (CTRL and GK). * Statistical difference, $p<0.05$.

2.2. Small intestine and colon microscopic evaluation

To assess whether gut remodeling occurs and follows a proximal-to-distal progression, as previously observed in rat T1D models, we measured both the mucosal and muscle layers in a T2D rat model. The histomorphometric evaluation of the small and large intestine of GK animals showed a higher thickness of the total intestinal wall of the duodenum, ileum, cecum and distal colon (DC) compared to controls (duodenum: 1089.02 ± 39.19 µm vs 864.19 ± 37.17 µm; ileum: 726.29 ±24.75 µm vs 498.76± 16.86 µm; cecum: 642.24 ±34.15 µm vs 500.97 ± 28.81 µm; DC: 1211.81 ± 51.32µm vs 831.71 ± 53.25 µm, respectively, $p<0.01$ for all). There was no difference between GK and control rats in histomorphometric evaluation of the jejunum and proximal colon (PC) (jejunum: 796,16 ± 43.86 µm vs 722.12 ± 28.75 µm and PC: 1060.18 ± 18.93 µm vs 1029.01 ± 59.84 µm, respectively, $p>0.05$ for both) (figure 3a).

The muscular layers of the intestinal wall of GK animals were increased in all segments except in the duodenum compared to controls (jejunum - longitudinal muscle (lm): 41.69 ± 2.80 µm vs 25.54 ± 2.28 µm, circular muscle (cm): 91.99 ± 5.03 µm vs 55.33 ± 3.73 µm; ileum - lm: 51.99 ± 2.90 µm vs 27.87 ± 3.14 µm, cm: 100.11 ± 5.96 µm vs 57.19 ± 5.38 µm; cecum - lm: 54.44± 5.33 µm vs 36.57 ± 3.15 µm, cm: 179,36 ± 10.84 µm vs 107.82 ± 8.09

μm; PC - lm: 77.70 ± 8.97 μm vs 42.52 ± 1.87 μm, cm: 212.03 ± 13.73 μm vs 146.03 ± 11.12 μm; DC - lm: 83.31 ± 6.54 μm vs 46.04 ± 3.51 μm, cm: 283.40 ± 33. 86 μm vs 164.43 ± 3.51 μm, respectively, $p<0.05$ for all; duodenum - lm: 43.81 ± 2.67 μm vs 35.12 ± 4.30 μm, cm: 99.36 ± 7.80 μm vs 78.08 ± 9.93 μm, respectively, $p>0.05$) (figure 3b). Submucosal values were consistent across all portions, except for the ileum in GK rats, where an increase was observed (GK: 41.73 ± 2.9 μm vs CTRL: 28.04 ± 4.38 μm). The mucosa was only increased in the duodenum (GK: 892.48 ± 31.21 μm vs CTRL: 710.60 ± 24.82 μm), ileum (GK: 532.46 ± 15.87 μm vs CTRL: 385.66 ± 24.20 μm) and DC (GK:765.84 ± 16.86 μm vs CTRL: 566.01 ± 44.33 μm) of GK rats compared with controls, while the jejunum (GK: 630.34 ± 49.26 μm vs CTRL: 615.97 ± 30.80 μm), cecum (GK: 354.70 ± 24.00 μm vs CTRL: 292.72 ± 30.77 μm) and PC (GK: 728.53 ± 45.79 μm vs CTRL: 808.19 ± 51.10 μm) presented similar results in both GK and control animals (figure 3b). Additionally, in the epithelial layer villi length and crypt depth were also increased in the duodenum and ileum of GK rats (supplementary file 1).

In figure 4, representative images of both control (CTRL) and GK animals are displayed, encompassing all the studied sections. These images provide a comprehensive visual comparison, highlighting the differences in each portion analyzed.

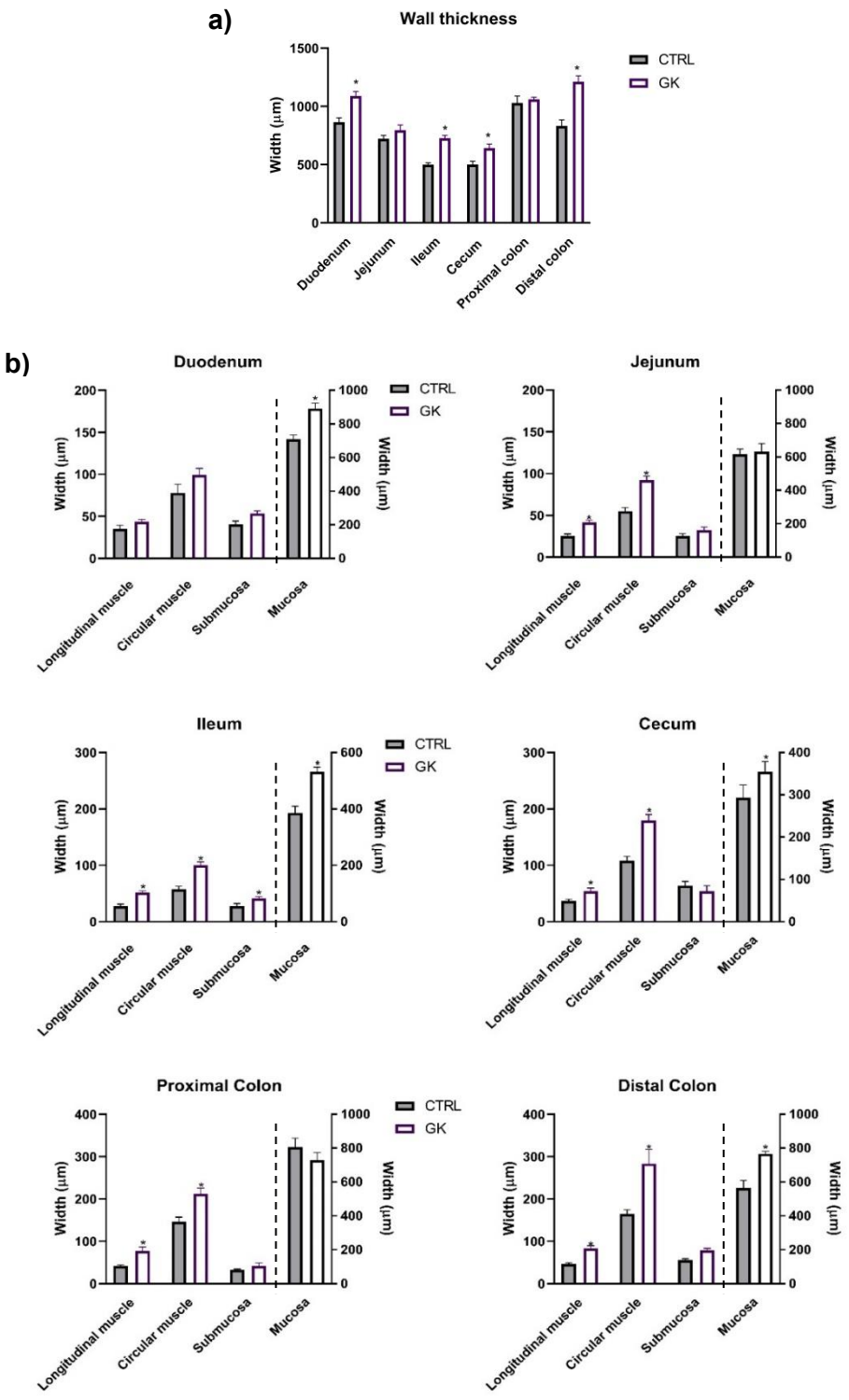

Figure 3 - Morphometric evaluation of intestinal segments (duodenum, jejunum, ileum, cecum, proximal colon, and distal colon) of control (CTRL, n=5) and GK diabetic rats (GK, n=6); a) total wall thickness (µm) of each intestinal segment; b) thickness (µm) of the intestinal layers (longitudinal muscle, circular muscle, submucosa and mucosa) of duodenum, jejunum, ileum, cecum, proximal colon, and distal colon) . Values are presented as mean ± SEM and a 2-way ANOVA followed by an unpaired t test with Welch's correction was used to compare the two experimental groups (CTRL and GK). * Statistical difference $p<0.05$ vs correspondent control. Unpaired t test with Welch's correction was used to compare the two experimental groups.

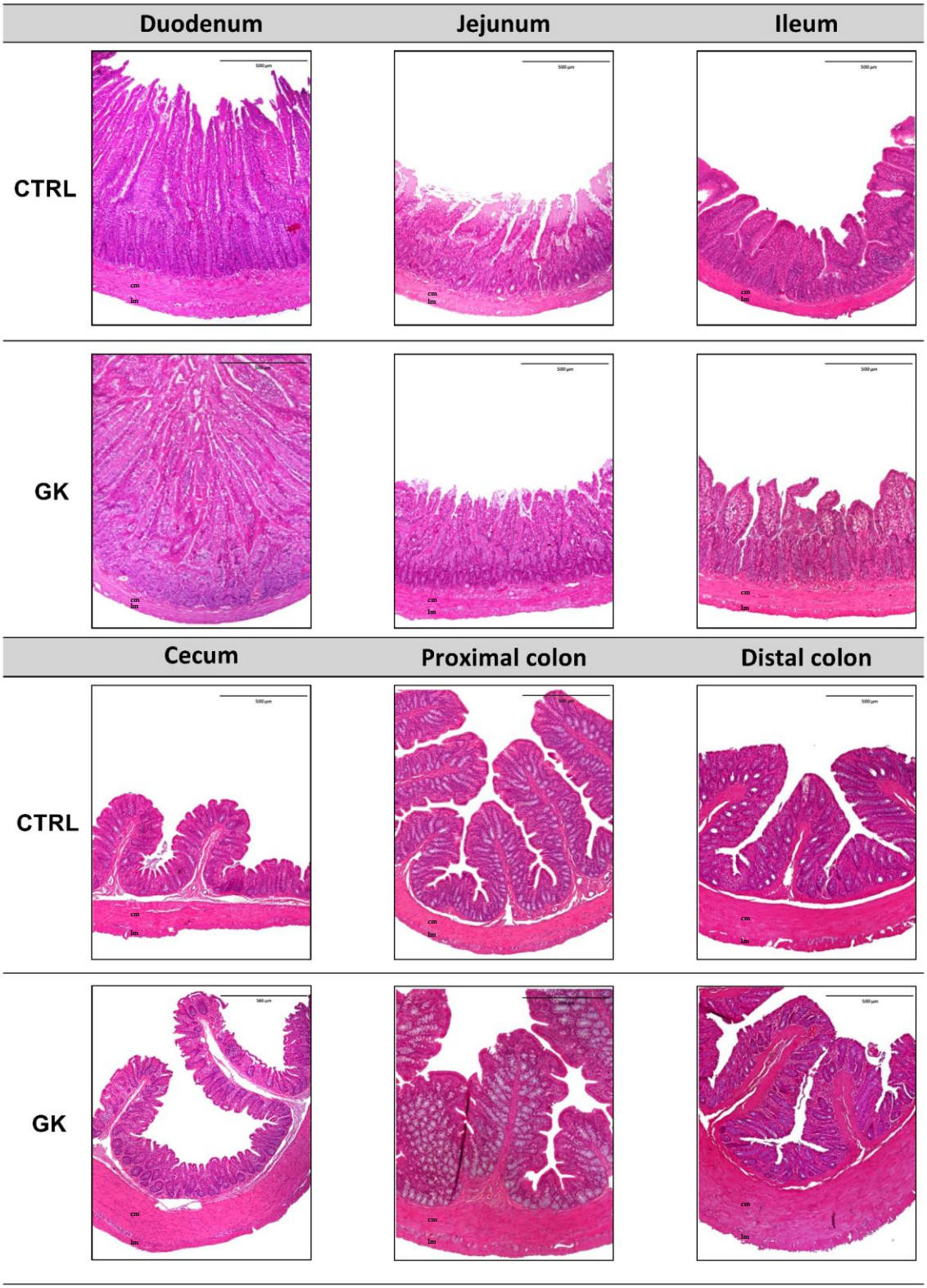

Figure 4 - Representative microscopic photographs of duodenum, jejunum, ileum, cecum, proximal colon and distal colon of control (CTRL) and GK rats (GK) stained with hematoxylin and eosin, captured using a 40x magnification. Longitudinal muscle (lm) and circular muscle (cm) were identified in all images.

Then collagen deposition was measured to evaluate potential tissue remodeling and fibrosis, conditions commonly linked to chronic hyperglycemia. These factors could explain the increased thickness of the muscular layers observed in the histomorphometric analysis. Masson's trichrome and periodic acid–Schiff (PAS) stains were assessed by an experienced pathologist blinded to the experiments. Interestingly, qualitative evaluation revealed no discernible differences between the control and GK diabetic animals. This suggests the absence of collagen deposition and no meaningful disparity in the proportion of carbohydrate macromolecules, such as glycogen, between the GK and control animal groups. Representative microscopic photographs of the colon with both staining techniques are shown in figure 5.

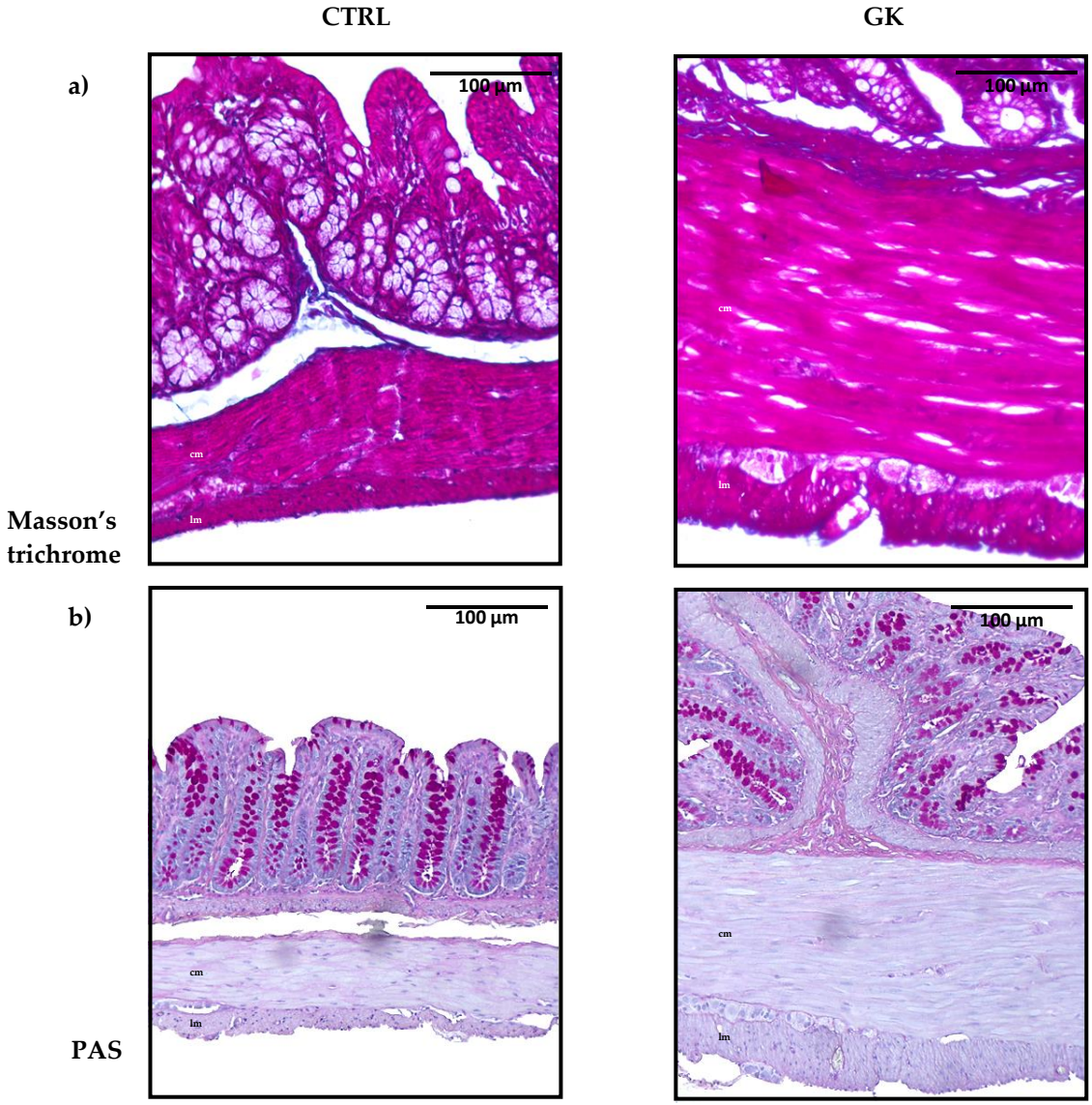

Figure 5 - Representative microscopic photographs of the colon of control (CTRL) and GK rats (GK) stained with Masson's trichrome (a) and periodic acid–Schiff (PAS) (b), captured with a 100x magnification. Longitudinal muscle (lm) and circular muscle (cm) were identified in all images.

2.3. Smooth muscle cells density in the muscular layers

Smooth muscle cell density was quantified in response to the negative results from Masson's trichrome staining, to determine whether an increase in density might be linked to smooth muscle hypertrophy. The number of nuclei of smooth muscle cells (SMC) was lower in GK in all portions studied compared to controls except for the duodenum (jejunum: 15.42 ± 0.89 vs 18.75 ± 0.1; ileum: 12.23 ± 0.80 vs 15.35 ± 0.57; cecum: 9.65 ± 0.65 vs 13.50 ± 0.67; PC: 14.90 ± 0.80 vs 18.68 ± 0.52; DC: 10.06 ± 0.64 vs 13.25 ± 0.51. respectively, $p<0.02$ for all; duodenum: 17.58 ± 0.74 vs 19.08 ± 0.31, respectively, $p>0.05$) (figure 6a). Representative microscopic images focusing on the muscular layers are depicted in Figure 6b.

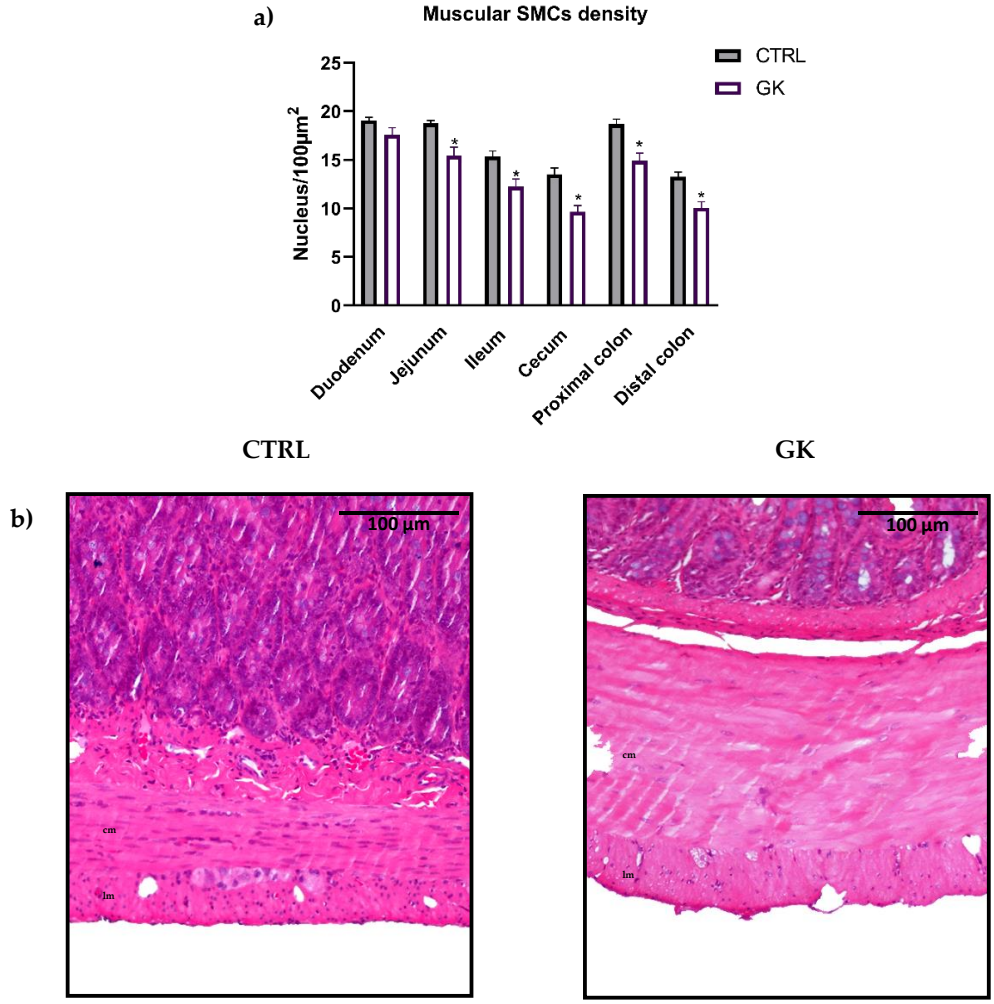

Figure 6 – a) Morphoquantitative analyses of the density of smooth muscle cells (SMC) in the muscular layers of duodenum, jejunum, ileum, cecum, proximal and distal colon of control group (CTRL, n=5) and GK diabetic rats (GK, n=6). Data is expressed as the mean ± SEM and comparisons between the two groups were made using Student's t- test. * Statistical difference, $p<0.05$. b) Representative microscopic photographs of the muscle layers of distal colon of control (CTRL) and GK rats (GK) stained with hematoxylin and eosin, captured with 100x magnification. Longitudinal muscle (lm) and circular muscle (cm) were identified in both images.

2.4. Neuronal density in the myenteric plexi

Neuron density was also assessed, based on the findings of Honoré *et al.* (2011), which suggested that neuronal loss could contribute to increased colonic thickness, potentially due to the greater force required for motility. Both smooth muscle cell and neuron densities are critical for maintaining proper gastrointestinal motility and function, which may be compromised by prolonged diabetes.

The neuronal density in the myenteric plexus was lower in the GK group, when compared to control rats (figure 7a). The number of nucleus *per* mm^2 was statistically lower in diabetic animals compared to controls in all portions studied (duodenum: 444.95 ±13.97 vs 540.54 ± 21.47; jejunum: 461.65 ± 31.78 vs 562.62 ± 10.86; ileum: 396.36 ± 12.73 vs 546.63 ± 15.94; cecum: 363.81 ± 17.74 vs 440.65 ± 24.82; PC: 382.36 ± 12.34 vs 511.90 ± 11.85; DC: 352.65 ± 27.94 vs 491.03 ± 21.47, respectively, $p<0.05$ for all). Representative microscopic images focusing on the myenteric plexus are depicted in Figure 7b.

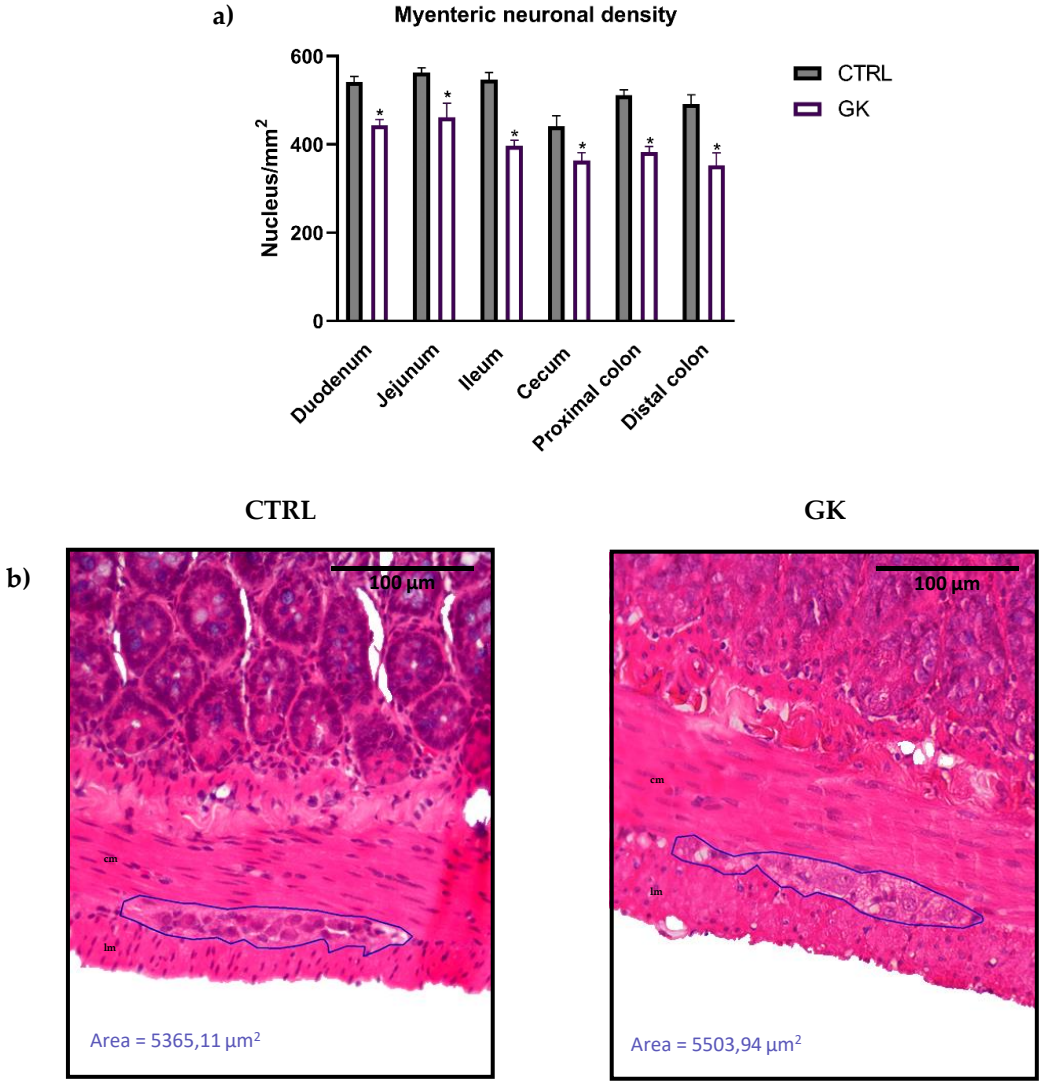

Figure 7 – a) Morphoquantitative analyses of the neuronal density in de myenteric plexus of duodenum, jejunum, ileum, cecum, proximal and distal colon of control group (CTRL, n=5) vs GK diabetic rats (GK, n=6). Data is

expressed as the mean ± SEM and comparisons between the two groups was made using Student's t- test. * Statistical difference, $p<0.05$. b) Representative microscopic photographs of the myenteric plexus proximal colon of control (CTRL) and GK rats (GK) stained with hematoxylin and eosin, captured with 100x magnification. Longitudinal muscle (lm) and circular muscle (cm) were identified in both images.

2.5. Total GSH and GSSG quantification

To investigate the potential causes of decreased neuron density observed in the myenteric plexus of GK rats, we decided to measure GSH levels as an indicator of oxidative stress, a critical factor in the development of diabetic complications. The results of the total glutathione quantification showed a decrease of tGSH in diabetic animals compared to controls in all portions studied except the duodenum (in nmol tGSH/mg protein, jejunum: 1.01 ± 0.06 vs 2.11 ± 0,03; ileum: 0.92 ± 0.11 vs 2.17 ± 0,15; cecum: 0.91 ± 0.01 vs 2.24 ± 0.15; PC: 0.94 ± 0.06 vs 2.09 ± 0.12; DC: 0.87 ± 0.02 vs 2.44 ± 0.19, respectively, $p<0.02$ for all; duodenum: 1.11 ± 0.20 vs 1.17 ± 0.07, respectively, $p>0.05$) (figure 8a). However, the quantification of GSSG revealed comparable values between GK rats and controls across all studied portions ($p>0.05$ for all) (figure 8b). Regarding the GSH/GSSG ratio, it was observed a decrease in all portions of GK diabetic rats compared to controls (duodenum: 6.04 ± 0.24 vs 8.28 ± 0.32; jejunum: 4.77 ± 0.31 vs 9.39 ± 1.31; ileum: 4.62 ± 0.52 vs 10.27 ± 1.20; cecum: 3.94 ± 0.31 vs 10.84 ± 1.22; PC: 4.84 ± 0.53 vs 9.15 ± 0.16; DC: 4.35 ± 0.47 vs 8.83 ± 0.62, respectively, $p<0.05$ for all) (figure 8c).

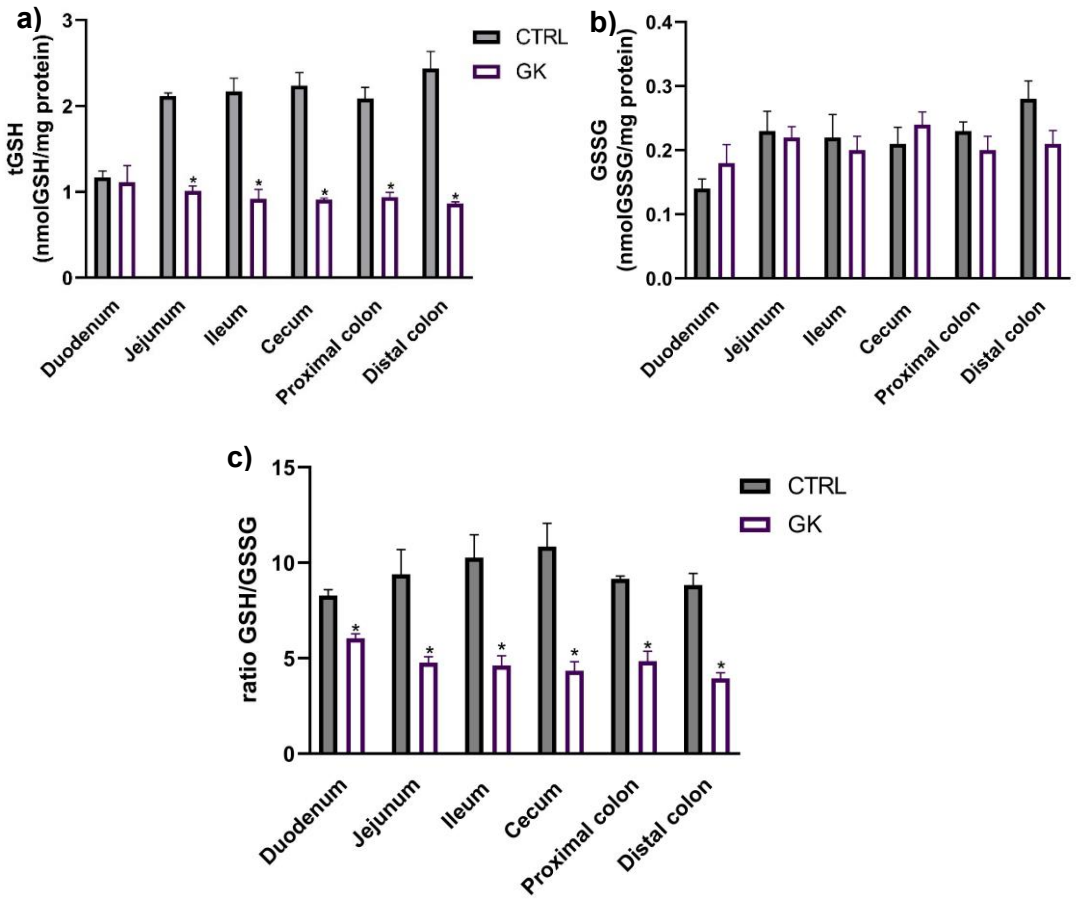

Figure 8 – Glutathione evaluation of intestinal segments (duodenum, jejunum, ileum, cecum, proximal colon, and distal colon) of control (CTRL, n=5) and GK diabetic rats (GK, n=6); a) Total glutathione (tGSH) quantification (nmol GSH/mg protein); b) oxidized glutathione (GSSG) quantification (nmol GSSG/mg protein); c) ratio GSH/GSSG. Values are mean ± SEM and an unpaired t test with Welch's correction was used to compare the two experimental groups (CTRL and GK). * Statistical difference $p<0.05$ vs correspondent control.

3. Discussion

This study presents a novel approach by examining local glutathione levels and morphometric changes in entire gut of GK rats. To the best of our knowledge, this is the first comprehensive study to analyze histomorphometry and quantify tGSH, GSSG, and the GSH/GSSG ratio across the entire gut, including the proximal and distal colon, all intestinal segments, and the cecum, in a T2D model. The assessment of local GSH provides a more localized and precise evaluation of oxidative stress within the gastrointestinal tract. This is a novel approach in diabetic animal models, offering new insights into how different regions of the intestine respond to diabetes-related oxidative stress.

In this work, 21-week-old male GK rats exhibited reduce weight compared to their Wistar counterparts while presenting higher food intake. They also showed fasting basal

hyperglycemia and impaired insulin sensitivity when compared to the control group. It was already shown that GK animals fail to accumulate body fat despite their higher calorie consumption and that adipose tissue is a major contributor to the differential weight in these animals [33]. These changes are due to an impairment in pre-adipocyte differentiation into mature adipocytes, leading to a defect in triglycerides storage [33]. Therefore, these findings align with the expectations for the GK model since the average body weight of GK rat is expected to be 10-30% less than that of their age-matched control Wistar rat [34]. Basal hyperglycemia has also been documented in GK rats, often manifesting as early as 3 weeks of age [35]. At birth, the β-cell mass of the GK rat is already severely reduced compared to that of the Wistar rat [36] and in adult GK rats the β-cell mass is usually reduced up to 60% with markedly decreased insulin secretion [37, 38] which explains the early hyperglycemia. Insulin resistance, another well-documented trait of this genetic model of T2D [15, 39] also aligns with the findings of our study. Although inadequate β-cell proliferation in early life is a limitation as it relates to the human condition, other characteristics are consistent with descriptions in the literature and validate GK rats as a non-obese T2D animal model [34, 40].

Histomorphometric changes in the gut of other animal models of diabetes were already described [21, 22, 24, 41, 42], but this is the first study to comprehensively examine the gut from the duodenum to the distal colon. This approach was chosen in order to ascertain whether we would observe a similar proximal-to-distal progression of the disease as previously described in models of T1D [22, 43]. In the GK rat, a T2D model, we did not observe such a pattern. Gut remodeling appears to occur in both mucosa and muscle layers, in different regions of the gut. The increase in the mucosa layer has been reported, and it was suggested that it was a mechanism to augment the absorptive surface area and functional capacity of the intestine [44]. Hyperphagia occurs in almost every model of diabetes and has also been suggested as a contributor to the increase in the thickness of intestinal mucosa [43]. However, it appears that the hyperglycemic state itself is sufficient to promote significant mucosal growth independent of food intake [45]. This was reinforced by another study where insulin administration prevented the marked increase in the intestinal epithelial cell proliferation rate of type 1 diabetic rats, resulting in reduced intestinal mucosal growth compared to non-treated diabetic animals [46]. Adachi et al. showed that GK rats also exhibited intestinal hyperplasia, possibly due to the increased expression of transcription factors and proteins involved in cell regeneration, differentiation, and/or proliferation [47]. The increase in the muscle layers of the gut was also already reported in several animal models of diabetes [21, 24, 41, 48]. In our study, the increase in the thickness of muscular layers may be at least partially attributed to hypertrophy of SMC since we observed a decreased density, rather than an increase. This finding was consistent across

all portions examined (not reaching statistical significance in the duodenum). SMC hypertrophy was already described by Horváth et al., who related this alteration with contractile protein actin and myosin increase in diabetic patients [17].

The myenteric plexus is located between the circular and the longitudinal muscular layers and is the main responsible for GI motility control [16, 49]. In contrast to the findings of Pereira et al. [24], who did not observe a significant difference in the number of myenteric neurons per unit area between GK animals and controls, our study revealed a decrease in the density of myenteric neurons in diabetic animals. It is worth noting that our animals were older compared to the study by Pereira et al. [24] therefore, the duration of diabetes may play a role in the development of these alterations. Additionally, several authors also reported changes in the number and size of myenteric neurons throughout the entire GI tract, including the stomach [43], duodenum [50], jejunum [51], ileum [49], cecum [52] and colon [53], in both type 1 (streptozotocin-induced diabetic rats) [54] and type 2 D (diabetic mice consuming a high-fat diet) [55]. It seems that the neuronal population of the submucosal plexus may be more susceptible to degenerative changes induced by diabetes compared to the myenteric plexus [56]. The mechanisms underlying neuronal loss encompass increased apoptosis, elevated levels of Advanced Glycation End products (AGEs) and Receptor of Advanced Glycation End products (RAGEs), reduced nerve growth factor levels, and heightened oxidative stress [51, 53, 57].

These changes in the morphology of the small intestine and colon result in biomechanical alterations such as loss of matrix elasticity and contractility, impairing both contraction and relaxation responses, which are fundamental for maintaining normal GI motility [32, 58]. This leads to impaired intestinal sensory function and reduced intestinal motility [41, 59, 60], while increased thickness of the mucosa can affect digestion and absorption [61]. The neuronal change can further lead to improper gut motility, retrograde colonic movements, altered secretions, and even increased pain stimuli [62, 63]. These alterations may provide insight into the common GI symptoms observed in diabetic patients [21].

Oxidative stress results from an imbalance between the production of ROS and antioxidant defenses and has already been implicated in gastrointestinal complications of diabetes [64, 65]. Given that glutathione serves as the body's primary antioxidant playing a crucial role in combating oxidative stress [66] and that previous studies showed that hyperglycemia-related oxidative stress was a primary inducer of neurological damage [16], we chose to quantify GSH levels locally. To the best of our knowledge, this is the first study to comprehensively evaluate tGSH and GSSG levels and GSH/GSSG ratio across all sections of the gut in diabetic animals. In this work, we observed a decrease in tGSH levels in all examined segments of the gut, except for the duodenum. Furthermore, while the levels of GSSG were comparable between diabetic and control animals, the ratio of GSH to GSSG

was significantly lower in diabetic animals (including in the duodenum), indicating increased levels of oxidative stress. The reduction in the GSH/GSSG ratio can result from either a decrease in free GSH levels or an increase in GSSG levels. In this study, the observed decrease in the GSH/GSSG ratio in GK rats is primarily due to a reduction in GSH levels in GK animals compared to controls, as there were no significant differences in GSSG levels between the two experimental groups. Chandrasekharan et al. conducted the first and only quantification of GSH but only in the diabetic colon as an indicator of oxidative stress, wherein they also observed a decrease in GSH levels associated with neurological damage and motor dysfunction [32]. These are likewise consistent with findings in individuals with T2D, who have been reported to exhibit lower blood GSH values [67, 68]. Also, the depletion of GSH observed in a streptozotocin-induced model of diabetes has been shown to cause cardiac damage and cardiomyocyte apoptosis [69]. In another study, a decrease in GSH levels was observed in vascular smooth muscle cells, which was attributed to the depletion of glutathione precursors, particularly cysteine, which is a rate-limiting substrate in new glutathione synthesis [70]. Sekhar *et al.* described that the principal cause of oxidative stress in T2D is a deficiency of glutathione, primarily stemming from reduced synthesis due to limited availability of the precursor amino acids cysteine and glycine and that supplementation of these precursors through dietary means can restore the synthesis of glutathione, consequently leading to a significant reduction in oxidative stress and markers of oxidant damage [71]. Furthermore, in individuals with type 2 diabetes, increased levels of transforming growth factor beta (TGF-β) were observed in their plasma samples. This cytokine is known to reduce the expression of the catalytic subunit of glutamine-cysteine ligase, which also helps to explain why GSH levels decrease in these individuals [72].

The alterations observed in the GSH and GSSG concentration in our study led to a decrease of up to 60% in the GSH/GSSG ratio in GK rats compared to controls. This reduction closely mirrors findings reported by Calabrese et al., who observed a 68% decrease in plasma GSH levels in T2D patients compared to control subjects [73]. The decrease in the plasma GSH/GSSG ratio not only correlates with heightened oxidative stress but also appears to adversely affect glucose availability and homeostasis, thereby exacerbating the diabetic condition [74, 75]. Additionally, oxidative stress is known to play a critical role in the pathogenesis of various diabetic complications, including neuropathy, nephropathy, and retinopathy [27]. Furthermore, oxidative stress has also been identified as a significant contributor to gastrointestinal dysmotility, including post-operative ileus and diabetic gastroparesis [64]. Maintaining a balanced GSH/GSSG ratio is essential for protecting cells from oxidative damage and ensuring proper metabolic functioning [76]. Therefore, our findings highlight the importance of addressing oxidative stress when studying gastrointestinal complications of diabetes.

The results of histomorphometry and oxidative stress combined reveal an interesting pattern: all sections of the intestine showed signs of oxidative stress (indicated by a decreased GSH/GSSG ratio) and neuronal damage, but muscular remodeling was not observed in every portion. In fact, the duodenum displayed both oxidative stress and neuronal damage, but the muscular layers showed no remodeling. This raises an important question: does neuronal damage from oxidative stress occur before intestinal remodeling? These findings prompt further investigation into the sequence of events leading to gastrointestinal complications in diabetes.

In conclusion, we identified significant remodeling of the intestine and colon, along with marked alterations in the neuronal population of the myenteric plexus. The critical local deficiency of GSH, a key antioxidant, emerged as a central factor driving increased oxidative stress, which likely underlies the observed structural and neuronal damage in the gut of GK rats. Furthermore, the reduced GSH/GSSG ratio further underscores the oxidative stress in the examined gut regions. This data sheds some light on the complex interplay between diabetes and gastrointestinal adjustments, offering new insights that could enhance our understanding and management of diabetic complications (figure 9).

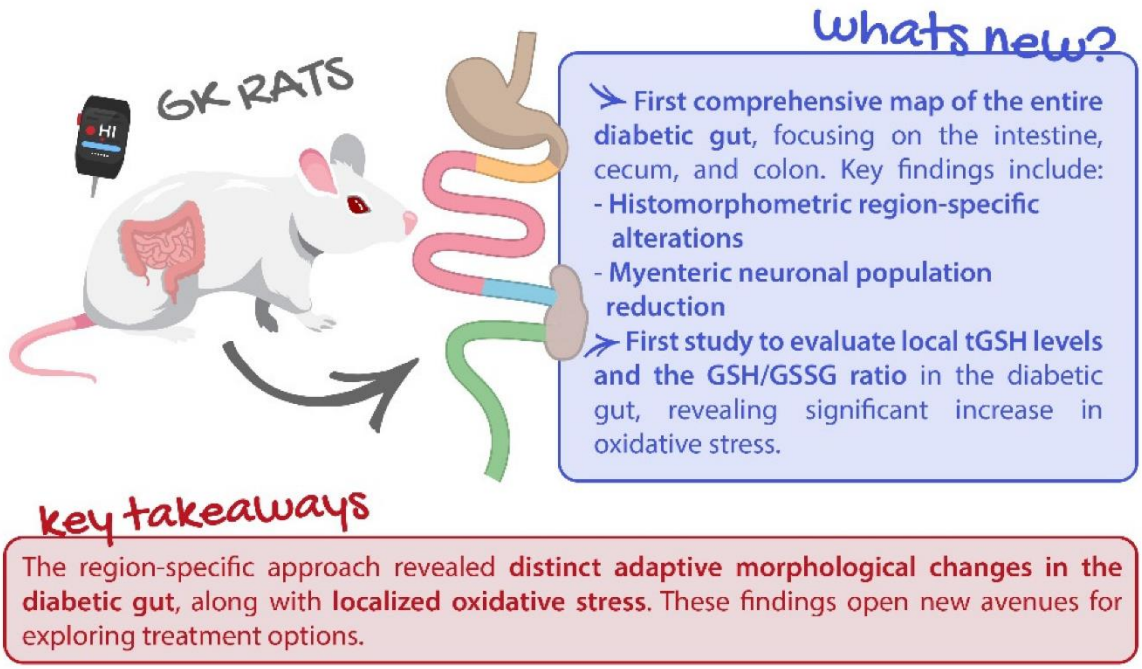

Figure 9. This study combines histomorphometry with glutathione assessments, providing a dual layer of analysis that allows for a more comprehensive understanding of tissue health and oxidative damage across different diabetic gut regions.

4. Materials and Methods

4.1. Animals

Non-obese type 2 diabetic GK male rats (n=6), 20-21 weeks old, were obtained from the breeding colonies of the Faculty of Medicine, University of Coimbra. Wistar Han rats (n=5) from the same colony with comparable age were used as controls. Animals were kept under standard ventilation, temperature (22.0 ± 0.1 °C), relative humidity (52.0 ± 2.0%) and light (12 hours light/ dark cycle) with access to autoclaved tap water and food ad libitum (standard diet A03, SAFE, France). All procedures involving animals were previously approved by the local animal welfare commission (ORBEA 13/18) following the European Community guidelines for the use of laboratory animals (Directive 2010/63/EU) and performed by licensed users.

4.2. In Vivo Procedures and Sample Collection

Animals' body weight, caloric intake and blood glucose (6h fast, blood collected from the tail vein) were monitored for 2 weeks.

Intraperitoneal insulin tolerance tests (ITT, Humulin, Lilly®, 0.25 IU/kg) were performed after a 6h fast. Glycaemia evaluation was performed at 0, 15, 30, 60 and 120 min using a glucometer and test strips (Accu-Chek Aviva, Roche®, Basel, Switzerland) [77].

After a 6 hour fast, animals were anesthetized with an intraperitoneal injection of ketamine (Nimatek, Dechra®, 50 mg/kg) and xylazine (Sedaxylan, Dechra®, 6.6 mg/kg), and after blood collection were sacrificed by cervical displacement. The GI tract from the proximal part of the duodenum to the distal part of the colon was collected and weighed as previously described by our group [22].

4.3. Histological preparation and analyses

Samples (1cm long) of proximal duodenum (collected 2cm distal to pylorus), middle jejunum, distal ileum (collected 2cm cranial to the ileocecal junction), cecum, proximal colon (PC) and distal colon (DC) were collected and fixed in 4% formalin. All samples were dehydrated in consecutive 70%, 96% and 99% ethanol solutions and embedded in paraffin. Then, 3 µm-thick cuts were made perpendicularly to the mucosa using a microtome and mounted in sterilized glass slides. Finally, the sections were rehydrated in a series of graded ethanol (99, 96, 70%), washed in water, and stained with hematoxylin and eosin (H&E). Each section was evaluated under an optical microscope (Eclipse E600Miami, Nikon Instruments®, USA) and photographed in different representative regions (magnification of

40x and 100x). All stained samples were evaluated by an experienced veterinary pathologist who was blinded for the experimental groups. The thickness of the mucosa, submucosa, circular and longitudinal muscles was then measured, by the same research team member, using the free ImageJ® software. For each sample the layer thickness was measured randomly in twelve different locations, and then averaged. The measurements were only carried out in images where the entire intestinal wall could be observed. To evaluate collagen deposition in the extracellular matrix, the samples were stained with Masson's trichrome, and to measure the intracellular accumulation of glycogen, the Periodic Acid-Schiff (PAS) reaction was performed. All histologic samples were evaluated by an experienced veterinary pathologist.

4.4. Quantitative analysis of smooth muscle cells nuclei in the muscular layers

For each sample, twelve sections centered in the muscular layers were photographed (objective lens of 10x). For each section, an area of 50 µm X 200 µm (10000 μm^2) in the center of the photo was used for nuclei quantification per unit area. Only the nuclei of the SMC within the test area boundaries and those that touched the lines were counted. SMC nucleus density was expressed as the number of cells *per* mm^2 of muscular area.

4.5. Quantitative analysis of neuronal nuclei in the myenteric plexi

For each sample, three sections stained with H&E were observed and all myenteric plexi were photographed using 10x, 20x and 40x objective lenses when needed. The myenteric plexi were then outlined, and their areas were measured. The neurons' nuclei within all visible sections of the myenteric plexus were counted. Myenteric neuronal density was expressed as the number of cells per mm^2 of the plexus.

4.6. Total GSH and GSSG quantification

For total GSH (tGSH) and GSSG quantification, 1cm long samples of the proximal duodenum, middle jejunum, distal ileum, cecum, PC and DC were collected and 400 µL of perchloric acid 5% (w/v) was added. The tissues were homogenized and centrifugated at 16060 xg for 10 min at 4°C. The pellets were then saved for protein quantification at -20°C and the acidic supernatant was stored at -80°C until analysis.

The levels of tGSH and GSSG were measured using the DTNB-GSSH reductase recycling assay, following the modified Ellman's method [78]. Acidic samples were neutralized with 0.76 M potassium bicarbonate and then centrifuged (16060 g for 2 minutes at 4°C). The

same process was applied to GSH standards ranging from 0 to 15 µM. In 96-well plates, 100 µL of sample was mixed with 65 µL of reagent solution containing NADPH (0.63 mM) and DTNB (3.96 mM), prepared in phosphate buffer (71.5 mM Na2HPO4, 71.5 mM NaH2PO4, 0.63 mM EDTA). The mixture was incubated at 30°C for 15 minutes. Subsequently, 40 µL of glutathione reductase (10 U/mL in phosphate buffer) was added, and absorbance readings were taken at 415 nm for 3 minutes with 10-second intervals, using a Biotek PowerWaxe X spectrophotometer (Vermont, USA). The tGSH and GSSG levels were normalized to protein levels and expressed as nmol/mg of protein. The GSH/GSSG ratio was calculated using the following formula:

$$GSH/GSSG = (tGSH - 2 \times GSSG)/GSSG$$

4.7. Protein quantification

The pellets described in the previous section were dissolved in 0.5 M NaOH, and an albumin stock solution was prepared with concentrations ranging from 0.0625 mg/mL to 1 mg/mL. The pellets were homogenized, and protein levels were assessed spectrophotometrically using a microplate reader (Biotek-Powerwave HT®), following the method described by Lowry *et al.*, with measurements taken at a wavelength of 700 nm [79].

4.8. Statistical analysis

The GraphPad Prism 8.1.2 was used for statistical analysis of data. The unpaired Student's t test was used for comparison between 2 experimental groups (CTRL and GK) and data were expressed as mean ± SEM, where n refers to the number of experimental animals. The Shapiro-Wilk test was employed to assess the normality of the data. All datasets had $p > 0.05$ and were considered to have passed the normality test. To evaluate histological and oxidative stress data, a two-way ANOVA followed by an unpaired t test with Welch's correction was used to compare the two experimental groups. In all cases, a p value of less than 0.05 was used to identify a statistically significant difference.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/ijms252212115/s1.

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2.4. Differential effects of Losartan and Finerenone on diabetic remodelling, oxidative stress and ACE activity in the gastrointestinal tract of streptozotocin-induced diabetic rats

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Abstract

Gastrointestinal (GI) complications contribute significantly to morbidity in diabetic patients, yet the role of the local renin-angiotensin-aldosterone system (RAAS) in gut remodeling remains poorly understood. This study investigated histomorphometric changes, local oxidative stress, and alterations in circulating and tissue-specific angiotensin-converting enzyme (ACE) and ACE2 activities in streptozotocin (STZ)-induced diabetic rats. Additionally, we evaluated the effects of the angiotensin II type 1 receptor (AT1R) blocker, losartan, and the mineralocorticoid receptor antagonist, finerenone, on these alterations. Type 1 diabetes was induced in adult male Wistar rats (n=24) via a single STZ injection (55 mg/kg). Diabetic rats were treated daily with losartan (20 mg/kg; STZ-LOS, n=8) or finerenone (10 mg/kg; STZ-FIN, n=8), both mixed with peanut butter. Non-injected rats served as controls (CTRL, n=8). After 14 days, stomach, duodenum, jejunum, ileum, and colon samples were collected for histological analysis, total glutathione (tGSH) and oxidized glutathione (GSSG) quantification, and assessment of systemic and local ACE and ACE2 activities. Diabetic rats exhibited increased GI wall thickness, particularly in the muscular and mucosal layers, along with elevated tGSH levels and a reduced GSH/GSSG ratio, indicative of oxidative stress. Losartan effectively prevented these alterations, whereas finerenone had no significant effect. Circulating ACE and ACE2 activities were elevated in diabetic groups, but the ACE2/ACE ratio remained unchanged. Locally, ACE activity was increased in all gut segments of diabetic animals, while ACE2 was comparable to CTRL, leading to a decreased ACE2/ACE ratio, particularly in the jejunum and duodenum of diabetic animals. Neither treatment significantly modified local ACE or ACE2 activity. The Z-FHL/h-HL ratio exceeded 1 and was similar between portions, with exception of jejunum and duodenum of diabetic animals were this racio was decreased. These findings highlight the distinct effects of losartan and finerenone on diabetes-induced GI remodeling. Losartan prevented muscular hypertrophy and oxidative stress, emphasizing the role of AT1R-mediated mechanisms, while finerenone had no effect, suggesting mineralocorticoid receptor blockade alone is insufficient. Discrepancies between systemic and local RAAS activity further underscore its complex regulation in diabetes. AT1R blockers like losartan may offer therapeutic benefits, warranting further investigation.

1. Introduction

Diabetes is a serious, chronic disorder that has become one of the fastest-growing global health emergencies of the 21st century[1]. There are two main forms of diabetes: type 1 (T1D) and type 2 (T2D). Although T2D is vastly more common, T1D is usually more serious, as it is caused by the destruction of pancreatic β-cells, leading to an absolute insulin deficiency[2,3]. Given the widespread prevalence and significant impact of diabetes, alongside the indispensable role of animal models in advancing scientific knowledge, the significance of utilizing such models for studying the disease's aetiology and testing novel antidiabetic medications becomes evident[4]. Streptozotocin (STZ)-induced diabetes is commonly used in research to replicate T1D in rodents due to its selective destruction of pancreatic β-cells, yielding structural, functional, and biochemical alterations similar to those seen in human diabetic patients[5].

Gastrointestinal (GI) complications of diabetes are highly prevalent and constitute a significant cause of morbidity, affecting up to 75% of diabetic patients, which influence their health status and quality of life[6,7]. However, awareness of these complications among physicians is often limited, with scant knowledge and treatment options available[8,9]. Diabetic intestinal dysfunction appears multifactorial, involving advanced glycation end-product (AGE) accumulation, enteric nervous system (ENS) damage, impaired cholinergic neurotransmission, and smooth muscle fibrosis contributing to GI tract remodelling with reduced elasticity and impaired intestinal wall compliance[10]. These GI complications often lead to conditions like gastroparesis, enteropathy, and colonic disorders (e.g., chronic constipation and diarrhoea)[11]. Despite these alterations being described since 1971[12], the precise mechanism underlying diabetes-related GI complications remains far less understood compared to diabetic retinopathy or nephropathy, for example[13]. Consequently, many patients remain undiagnosed and untreated[14].

The renin–angiotensin-aldosterone system (RAAS) is best known for its role in cardiovascular and renal health, but it is also active in the GI tract, where its effector peptide, angiotensin II (Ang II), acts primarily via the Ang II type 1 receptor (AT_1R) to influence smooth muscle contraction in the intestine and colon[15,16]. Ang II is primarily generated from Ang I by angiotensin-converting enzyme (ACE), and promotes vasoconstriction, inflammation, and fibrosis (classic RAAS)[17]. Conversely, ACE2 metabolizes Ang II into Ang 1-7, which counteracts these effects. Additionally, ACE2 converts Ang I into Ang 1-9, later processed by ACE into Ang 1-7[18]. ACE2 has a single catalytic domain, whereas ACE has two (N- and C-domains) with distinct specificities: the C-domain primarily hydrolyses Ang I into Ang II, while the N-domain metabolizes Ang 1-7 into Ang 1-5 and other peptides[19,20]. The balance between ACE and ACE2 activities reflects RAAS regulation, orchestrating the

interplay between its classic and counter-regulatory pathways[21]. Moreover, the RAAS is no longer seen solely as a circulating hormonal system; it is now recognized as a local tissue system[22]. Various tissues, including the cardiovascular and renal systems and even the GI tract can synthesize key components of the RAAS, containing all components necessary for the production of Ang II[23]. Our group already described a decreased response to Ang II in the ileum and colon of STZ-induced diabetic rats[24]. There is a close relationship between diabetes and the RAAS, with RAAS contributing significantly to the development of several diabetic complications[25]. It has been shown, in diabetic patients, an over-activation of the classical RAAS with increased levels of expression of circulating and tecidual ACE, specially cardiovascular and renal systems, leading to the increased production of Ang II and AT_1R activation, contributing to increased oxidative stress and fibrosis in several organs[26,27]. But, to date, no one considered the possible therapeutic role of RAAS on diabetic impairment of intestinal motility.

Various pathways contribute to tissue damage via RAAS, and one includes heightened oxidative stress, characterized by elevated levels of reactive oxygen species (ROS)[28]. Moreover, chronic hyperglycaemia is linked to decreased cellular levels of glutathione (GSH)[29]. GSH is the most powerful antioxidant in the organism; it undergoes oxidation to GSSG (glutathione disulfide) through reactions catalysed by GSH-peroxidase. GSSG is subsequently regenerated back to GSH by GSH-reductase, using NADPH as a cofactor. This dynamic interconversion between GSH and GSSG is crucial for regulating redox-dependent cell signalling[30]. The excessive production of ROS prompts glutathione peroxidases to form GSSG thus altering the ratio between GSH and GGSG[31]. Maintaining an optimal ratio of GSH to GSSG within the cell is crucial for survival and a decrease in this ratio may be used as a marker of oxidative stress[32]. Oxidative stress and ROS formation are already described as being markedly increased by uncontrolled hyperglycaemia[33]. Furthermore, oxidative stress has already been identified as a significant contributor to gastrointestinal dysmotility, including post-operative ileus and diabetic gastroparesis[34]. Also, our group recently showed a lower total GSH (tGSH) and GHS/GSSG ratio in the gut of a long-term model of T2D[35].

Given the limited understanding of local RAAS in gut remodelling and even less in diabetic conditions, this study aimed to evaluate the preventive effects of losartan, an AT_1 receptor blocker (ARB), and finerenone, a selective mineralocorticoid receptor antagonist (MRA), on GI remodelling and oxidative stress in STZ-induced diabetic rats. Additionally, we aimed to assess the ACE/ACE2 activity balance in the serum and GI tract, to characterize the circulating and local RAAS.

2. Material and methods

2.1. Animals and housing

This project was approved by the institutional ICBAS-UP animal welfare body (P515/2024). Thirty-two male Wistar rats, aged 10 to 12 weeks and weighing 250–350g, were used in this study. Animals were housed at the ICBAS-UP rodent facility, where they were maintained under a 12-hour light/dark cycle with controlled ventilation, temperature (20–24ºC), and relative humidity (40–60%). Each pair of rats was housed in Sealsafe Plus GR900 Tecniplast® cages with appropriate bedding (Corncob Ultra 12, Ultragene) and environmental enrichment, including nesting paper, paper tunnels, and a mixture of cereal seeds and flakes. All rats had free access to autoclaved water (two bottles *per* cage) and a laboratory rodent diet (4 RF21, Mucedola S.r.l., Italy).

2.2. Diabetes induction

Animals were randomly chosen to be allocated to four experimental groups. On the day of DM induction (d0), the rats were fasted for 4 hours with free access to autoclaved tap water. The STZ solution (S0130, Sigma-Aldrich) (55 mg/ml in citrate buffer pH 4.5) was prepared just prior to the injection, since a freshly prepared solution is considered to be more effective[36]. Diabetes was induced by a single intraperitoneal injection of 55 mg/kg of STZ[36], under the analgesic effect of tramadol (Tramal® oral suspension, 100 mg tramadol/ml, Grünenthal; 20 mg/kg, PO), administered moments before. Rats maintained *ad libitum* access to water and food through the remaining protocol. Animals were considered diabetic if 48h after STZ injection their blood glucose was ≥ 250mg/dL. Glycemia was evaluated using a GlucoMen Areo GK glucometer from Menarini Diagnostics® (small sample size < 0.6 µL blood) and compatible test stripes. The blood glucose level of diabetic rats was measured by puncturing one of the tail veins at day 0 (control value), day 2 (to confirm or discard diabetes), day 7 and day 14. Eight diabetic rats were voluntarily orally treated with Losartan (20mg/kg) (STZ+LOS group) and another eight were voluntarily orally treated with finerenone (10mg/kg) (STZ+FIN group), both mixed with peanut butter from the day of induction until the end of the protocol. Eight diabetic animals remained untreated (STZ group). Different animals of similar age and body weight (*n*=8), that did not undergo any of these procedures, were used as controls (CTRL group).

2.3. Animal monitorization and welfare evaluation

The animals used in this project were monitored daily, from 11:00 to 13:00 AM, throughout the entire protocol, with all observations recorded in individual evaluation tables (Annex 1).

Our assessment began in the maintenance room, where we evaluated coat appearance, piloerection, posture before and after a brief stimulus, signs of abdominal discomfort, and changes in breathing patterns. Next, in the observation room, with the box open inside the flow chamber, we reassessed these parameters and additionally evaluated the animals' hydration status. The Grimace Scale was also applied as part of the evaluation in order to evaluate pain signs[37]. Monitoring continued with the weighing of each animal, which was also conducted prior to the fasting period on day 0. Food and water intake were also measured daily. To ensure hygiene and welfare, cages were changed whenever they became excessively wet due to polyuria (a classical sign of diabetes), typically daily. For animal welfare considerations, they were always housed in pairs *per* cage.

2.4. Tissue harvesting

On day 14, CTRL, STZ, STZ+LOS and STZ+FIN rats were euthanized by isoflurane overdosage followed by decapitation, using a guillotine suitable for that species (Small Guillotine, Harvard Apparatus). Whole blood was collected and left to rest at room temperature for 2 hours. It was then centrifuged at 3000rpm for 20 minutes, and the serum was collected and stored at −80°C until analysis. The abdomen of each rat was opened, and the overall appearance of the viscera was evaluated, followed by the removal of the GI tract from stomach to the distal colon, sectioned just proximal to the pubic symphysis. The longitudinal length of the colon was measured, and then the GI tract was separated in four parts: cecum, colon, intestine and stomach. Cecum was discarded and then the remaining parts were gently cleaned of their content using Krebs-Henseleit solution (in mM: 118 NaCl; 4.8 KCl; 2.5 CaCl2.2H2O; 1.2 NaH2PO4.H2O; 1.2 MgSO4.7H2O; 25 NaHCO3; 0.02 Na2EDTA; 0.3 Ascorbic acid; 11 monohydrated glucose). A 1 cm portion of all parts of was opened through the non-mesenteric border and laid flat to measure the circumferential perimeter. The stomach was also opened to separate the glandular and forestomach parts, with the latter being discarded.

A 1 cm segment from colon, ileum, jejunum, duodenum and stomach of the CTRL, STZ, STZ+LOS and STZ+FIN rats was collected for ACE and ACE2 activity, protein quantification, histological examination, and total GSH (tGSH) and GSSG quantification (figure 1).

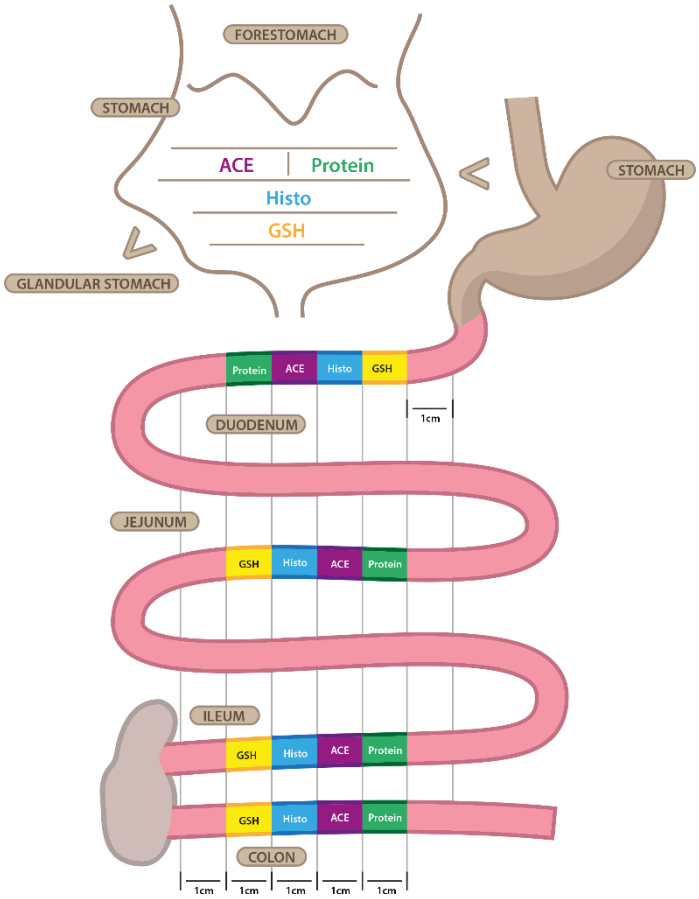

Figure 1 -Representative illustration of the localization of the different portions of stomach, duodenum, jejunum, ileum, colon, and removed in order to perform total glutathione and oxidized glutathione quantification (GSH), histopathology (histo), angiotensin converting enzyme (ACE) and ACE2 activity (ACE) and protein quantification (protein).

2.5. Histology

All samples were dehydrated in successive ethanol solutions (70%, 96%, and 99%) and embedded in paraffin. Then, 3 µm-thick sections were cut perpendicularly to the mucosa using a microtome and mounted on sterilized glass slides. The sections were subsequently rehydrated through a graded ethanol series (99%, 96%, 70%), washed in water, and stained with hematoxylin and eosin (H&E).

Each section was examined under an optical microscope (Nikon Instruments, Eclipse E600, Miami, FL, USA) and photographed in representative regions at 40x and 200x magnification. The thickness of the mucosa, submucosa, and circular and longitudinal muscle layers was measured by the same research team member using Nikon NIS-Elements software v6.10.01 (figure 2). For each sample, layer thickness was randomly measured at twelve different locations and averaged. Measurements were performed only on images where the entire intestinal wall was clearly visible.

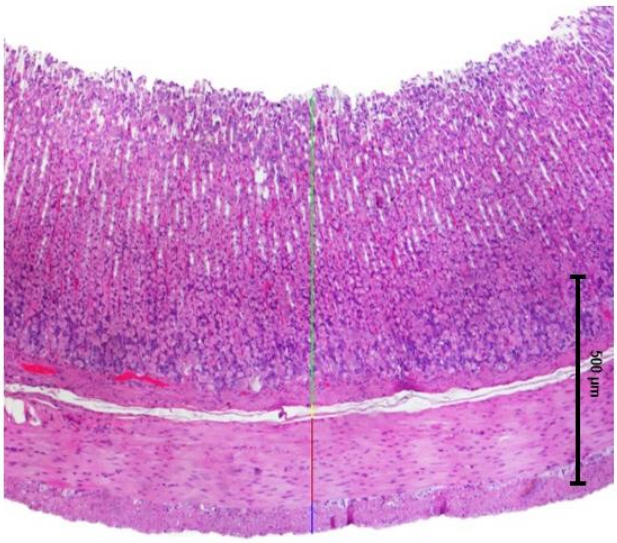

Figure 2 – Example of how the measurements in the histopathological images were conducted: blue – longitudinal muscle, red – circular muscle, yellow – submucosa, green – mucosa.

2.6. tGSH and GSSG quantification

For tGSH and GSSG quantification, collected samples were preserved in 1000 µL of 5% (m/v) perchloric acid. The tissues were homogenized in this solution and centrifuged at 13,000 rpm for 10 minutes at 4°C. The resulting pellets were stored at -20°C for subsequent protein quantification, while the supernatant was kept at -80°C until analysis. Total tGSH and GSSG levels were determined using the DTNB-GSSG reductase recycling assay, based on a modified Ellman's method[38]. Acidic samples were neutralized with 0.76 M potassium bicarbonate and centrifuged at 13,000 rpm for 2 minutes at 4°C. The same process was applied to total GSH standards (0–15 µM). In a 96-well plate, 100 µL of each sample was mixed with 65 µL of a reagent solution containing 0.63 mM NADPH and 3.96 mM DTNB, prepared in phosphate buffer (71.5 mM Na_2HPO_4, 71.5 mM NaH_2PO_4, 0.63 mM EDTA). The mixture was incubated at 30°C for 15 minutes, followed by the addition of 40 µL of glutathione reductase (10 U/mL in phosphate buffer). Absorbance was measured at 415 nm over 3 minutes, with readings taken every 10 seconds, using a Biotek PowerWave X spectrophotometer (Vermont, USA).

tGSH and GSSG concentrations were normalized to total protein content and expressed as nmol/mg of protein. The GSH/GSSG ratio was calculated using the following formula*:*

$$\text{GSH/GSSG} = \frac{tGSH - 2 \times GSSG}{GSSG}.$$

The pellets obtained in the previous step were dissolved in 0.5 M NaOH, and an albumin stock solution was prepared with concentrations ranging from 0.0625 mg/mL to 1 mg/mL. After homogenization, protein levels were quantified spectrophotometrically using a Biotek

PowerWave HT microplate reader, following the Lowry *et al.* method[39], with absorbance measured at 595 nm.

2.7. ACE and ACE2 activity

For the enzyme activity assay, the collected tissue segments (approximately 200 mg) were homogenized in 1 mL of buffer containing 100 mM sodium borohydride (pH 7.2), 340 mM sucrose, 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF) inhibitor. PMSF was initially prepared as a 200 mM concentrated solution and added to the homogenization tubes at the time of preparation to achieve a final concentration of 1 mM. Buffer preparation and sample homogenization were conducted on ice. The samples were then centrifuged at 3,000 rpm for 15 minutes at 4°C, and the supernatants were collected and stored at −80°C until analysis.

ACE activity was measured as previously defined[40]. Hippuryl-His-Leu (h-HL) and Z-Phe-His-Leu (Z-FHL) were used as substrates. Briefly, 10 µL of tissue homogenate was incubated at 37°C for 10 minutes with 200 µL of assay solution containing either 1 mM Z-FHL or 5 mM h-HL in 100 mM potassium phosphate buffer (pH 8.3), supplemented with 300 mM NaCl and 0.1 mM $ZnSO_4$. The enzymatic reaction was then stopped by adding 1.5 mL of 0.28 M NaOH. To allow the binding of o-phthaldialdehyde to the newly formed HL peptide, 100 µL of an o-phthaldialdehyde solution (20 mg/10 mL in methanol) was added, and the mixture was incubated at room temperature for 10 minutes, resulting in a fluorescent product. The reaction was terminated by adding 200 µL of 3 N HCl, followed by centrifugation at 3,000 rpm for 5 minutes at 4°C. The hydrolysis product HL was quantified fluorometrically (λ_excitation = 360 nm; λ_emission = 465 nm) using a SpectraMax Gemini EM microplate reader (Molecular Devices). ACE activity was expressed as global enzyme activity normalized to total protein concentration (nmol/min/mg of total protein) for intestinal tissue, and global enzyme activity for serum (nmol/min/mL).

ACE2 activity was measured using a fluorometric kinetic assay with 20 µM Mca-APK(Dnp) as the substrate (Cat. No. BML-P163-0001, Enzo Life Sciences, Inc.). Briefly, sample homogenates (10 µL for intestinal tissue or 5 µL for serum) were preincubated at 37°C for 5 minutes in a buffer containing complete Mini EDTA-free protease inhibitor (1 tablet per 10 mL buffer), 75 mM Tris, 1 M NaCl, 0.5 mM $ZnCl_2$, and 10 µM captopril (all from Merck®), at pH 6.5. Incubation was performed in the presence or absence of the selective ACE2 inhibitor MLN476(10 µM). The substrate was then added, and fluorescence was recorded every 2 minutes over 120 minutes (λ_excitation = 320 nm; λ_emission = 420 nm) using a SpectraMax Gemini EM microplate reader (Molecular Devices). Fluorescence values were obtained for two hours and calculations were performed based on a fluorescence standard

curve obtained with OmniMMP® fluorogenic control (Cat. No. BML-P127-0001, Enzo Life Sciences, Inc.), with the time point 0 serving as an internal blank. The maximum activity value for each sample was used and ACE2 activity was expressed as global enzyme activity normalized to total protein concentration (nmol/min/mg of total protein) for intestinal tissue, and global enzyme activity for serum (nmol/min/mL).

To evaluate the relative contribution between the activities of the N- and C- domains of ACE and between the activities of ACE2 and ACE in each sample, the following ratios were calculated and analysed: ACE-Z-FHL / ACE-h-HL activity ratio and ACE2/ACE-Z-FHL activity ratio. It has been reported that the Z-FHL/h-HL hydrolysis rate ratio for human ACE varies depending on the domain: both domains combined exhibit a ratio of approximately 1, the N-domain presents a ratio of 4.5, and the C-domain presents a ratio of 0.74[41].

Total protein quantification was performed using the Bradford method, with bovine serum albumin as the standard[42].

2.8. Statistical analysis

The GraphPad Prism® 8.1.2 (Graph Pad Prism Software, Inc.) was used for statistical analysis of data. The Shapiro–Wilk test test was employed to assess the normality of the data. All datasets that had $p > 0.05$ and were considered to have passed the normality test. To evaluate data with normal distribution, including histological and oxidative stress, between the four experimental groups (CTRL, STZ, STZ+LOS and STZ+FIN), an ordinary one-way ANOVA with multiple comparisons was used. Data was expressed as mean ± SEM, where *n* refers to the number of experimental animals. To evaluate data with a non-Gaussian distribution (ACE and ACE2 activities and respective racios) the Kruskal-Wallis test with multiple comparisons followed by Dunn's multiple comparisons test was used and data was expressed as median [95% confidence limits]. In all cases, a *p* value of less than 0.05 was considered to denote a statistically significant difference.

Sample size was decided using the free software Sample Size Calculator (©2024—ClinCalc LLC, https://clincalc.com/stats/samplesize.aspx).

3. Results

3.1. Animal monitorization

Before induction, basal glycemia of control and STZ-induced rats, treated and non-treated, was similar (CTRL: 122.75 ± 4.28 mg/dL vs STZ: 125.5 ± 5.10 mg/dL vs STZ+LOS: 119.13 ± 3.42 vs STZ+FIN: 122.88 ± 6.50, n=32, $p>0.05$) (figure 3). Induced rats had the initial glycemia increased to 529.5 ± 20.76 mg/dL (STZ), 494.25 ± 20.73 mg/dL (STZ+ LOS) and

523.38 ± 21.46 mg/dL (STZ+FIN) within 48 hours ($p < 0.0001$), while control rats glycaemia was roughly the same within 48 hours (131.63 ± 4.93 mg/dL; $p > 0.05$). At d7 and d14, almost all STZ rats presented with a glycemia above 600mg/dL with ketone bodies, while control animals presented glycemic values of 142.5 ± 4.96 mg/dL at the 14th day. The glucometer used was selected because it could quantify blood glucose values up to 600mg/dL, with values above this threshold being considered "HIGH". In this study, for graphic purposes we considered all "HIGH" values to correspond to 600mg/dL, a very high value which raises no doubt about the diabetic condition the animals were in throughout the protocol.

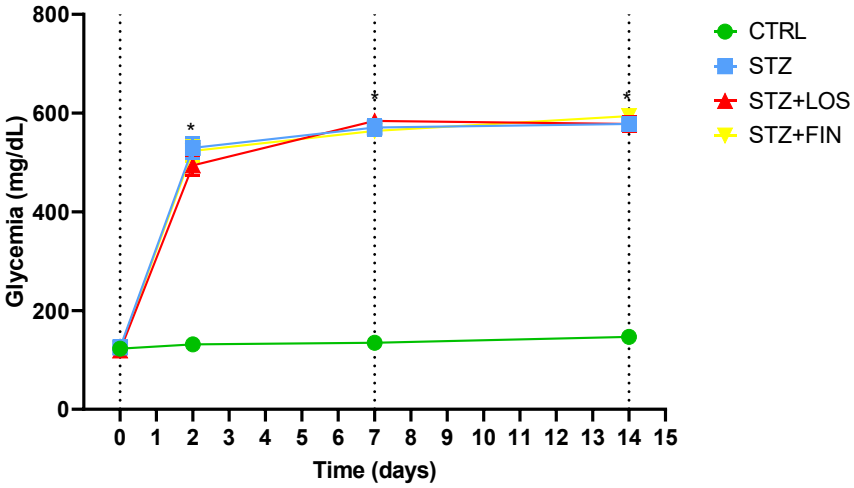

Figure 3 - Blood glucose concentrations of control (CTRL, n = 8), non-treated streptozotocin-induced (STZ, n = 8), streptozotocin-induced treated with losartan (STZ+LOS, n=8) and streptozotocin-induced treated with finerenone (STZ+FIN, n=8) rats measured on day 0, day 2, day 7 and day 14. Values are presented as mean ± SEM. * Statistical difference compared to CTRL, $p < 0.05$.

The parameters documented during the daily monitorization (% of body weight loss, water intake, food intake) are shown in Figure 4.

In the control group, rats progressively gained weight, their weight being 14.21 ± 2.78% higher by d14 than on d0 (before fasting). All diabetic rats had a consistent weight loss that was more pronounced on d2 (less 5% compared to the initial weight) and then maintained that relatively stable lower weight. The loss of body weight wasn't affected by any of the treatments applied. The % of body weight variation was 8.45 ± 2.53% (STZ), 4.52 ± 1.51% (STZ+LOS) and 8.2 ± 1.5% (STZ+FIN) lower at d14 when compared to the initial weight (before fasting) (figure 4).

The food intake was significantly higher in all three groups of diabetic rats than controls after the d4. STZ rats started the experimental protocol eating 44.67 ± 2.63 g in the first day, and progressively increased food consumption until the last day, when the intake was 88.33

± 11.41 g/cage/day. STZ+LOS rats started the experimental protocol eating 35.5 ± 3.71 g in the first day, and progressively increased food consumption until the last day, when the intake was 80.85 ± 3.94 g/cage/day. STZ + FIN rats started the experimental protocol eating 33.67 ± 7.26 g in the first day, and progressively increased food consumption until the last day, when the intake was 80.25 ± 5.15 g/cage/day. The control group maintained a constant food intake during the experimental time, with a mean consumption of 41.22 ± 3.38 g/cage/day. The four groups were always fed *at libitum*.

As expected, water intake was significantly higher in all diabetic groups comparing to controls that maintained a relatively constant water intake through all the experimental protocol: 56.44 ± 3.53mL/cage/day. All diabetic rats drank more water since d3, but their water intake increased progressively throughout the protocol, reaching values more than 7 times higher than those of control animals (at d14: STZ: 472.75 ± 19.69 mL/cage/day; STZ+LOS: 423 ± 30.08 mL/ cage/ day; STZ+FIN: 415 ± 22.29 mL/cage /day)

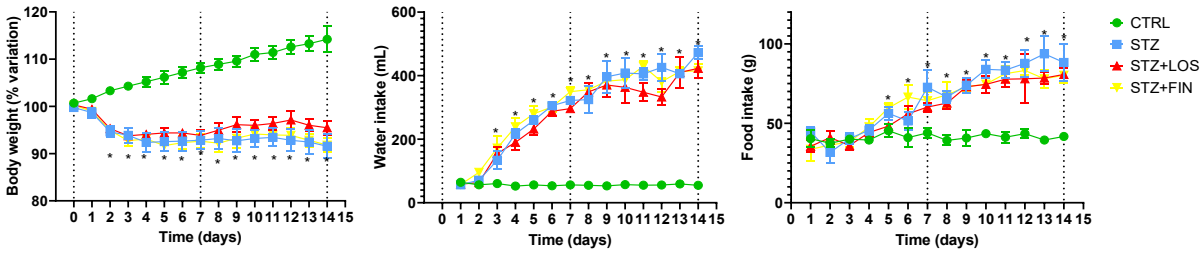

Figure 4 - Evaluation during the experimental protocol (14 days) in control (CTRL, n=8), streptozotocin-induced diabetic rats (STZ, n=8), STZ diabetic rats treated with losartan (STZ+LOS, n=8) and STZ diabetic rats treated with finerenone (STZ+FIN, n=8) of: % of body weight variation, water intake and food intake. Values are mean ± SEM. * Statistical difference to CTRL, $p < 0.05$.

3.2. Macroscopic evaluation

Colon length was significantly greater in the STZ group (23.35 ± 0.31 cm) compared to the CTRL group (17.38 ± 0.50 cm). Losartan prevented this increase, as the colon length in the STZ+LOS group (18.25 ± 0.73 cm) was comparable to the CTRL group. In contrast, finerenone treatment had no effect on that increase, with the colon length in this group (21.75 ± 0.75 cm) differing from the control group, but not from the STZ group (figure 5). We also measured the circumferential perimeter of all intestinal segments and found it to be significantly greater in the STZ group across all segments compared to the control group (in mm: duodenum: 14.17 ± 0.83 vs. 10.14 ± 0.51; jejunum: 15.5 ± 0.22 vs. 10.71 ± 0.29; ileum: 13.67 ± 0.42 vs. 10.43 ± 0.30; colon: 15.83 ± 0.31 vs. 12.00 ± 0.65; $p<0.05$ for all). Treatment with losartan effectively prevented this increase in all segments (duodenum: 11 ± 0.36; jejunum: 12.14 ± 0.51; ileum: 11.14 ± 0.40, $p<0.05$ for all compared to CTRL) except colon (13.7 ± 0.52, $p> 0.05$ compared to CTRL). In contrast, finerenone treatment did not

alter this increase (duodenum: 14.57 ± 1.13; jejunum: 14.85 ± 0.87; ileum: 14 ± 0.79; $p<0.05$ for all compared to CTRL), except for the colon (13.25 ± 1.03, $p>0.05$) (figure 5).

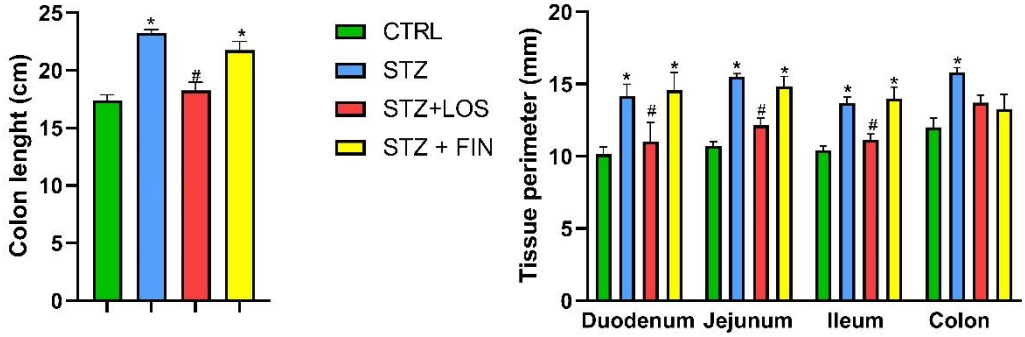

*Figure 5 - Macroscopic evaluation: colon length (cm) and tissue perimeter (mm) of duodenum, jejunum, ileum and colon of control (CTRL, n=8), streptozotocin-induced diabetic rats (STZ, n=8), streptozotocin-induced diabetic rats treated with losartan (STZ-LOS, n=8) and streptozotocin-induced diabetic rats treated with finerenone (STZ-FIN, n=8). Values are mean ± SEM. * Statistical difference ($p < 0.05$) to CTRL; #Statistical difference ($p < 0.05$) to STZ.*

3.3. Microscopic evaluation

The results of the microscopic evaluation of the intestinal segments from all groups were consistent with the macroscopic data previously described, revealing an increase in the thickness of the intestinal layers in the duodenum, jejunum, ileum, and colon of STZ animals compared to controls, and its prevention by losartan but not by finerenone.

Looking at the wall thickness as a whole, STZ portions showed an increased in the thickness compared to control in stomach (1216.28 ± 73.64 µm vs 1023.89 ± 13.38 µm), duodenum (1178.89 ± 99.59 µm vs 866.23 ± 69.34 µm), ileum (784.39 ± 26.65 µm vs 515.19 ± 20.01 µm) and colon (900.13 ± 55.43 µm vs 669.78 ± 47.06 µm), but not in jejunum (859.42 ± 53.80 vs 611.83 ± 83.16, $p>0.05$) . Treatment with losartan was only able to prevent this increase in the colon (724,27 ± 22.61 µm). Finerenone treatment did not have any effect on the wall thickness.

Both muscular layers (longitudinal muscle - LM and circular muscle - CM) were increased in all the portions studied in STZ animals compared to controls (LM, in µm: stomach: 121.30 ± 10.28 vs 75.64 ± 7.57; duodenum: 61.78 ± 4.57 vs 39.44 ± 2.1; jejunum: 60.89 ± 3.92 vs 36.37 ± 1.37; ileum: 63.60 ± 5.18 vs 35.72 ± 1.69; colon: 66.29 ± 14.91 vs 38.75 ± 1.07; CM, in µm: stomach: 315.56 ± 23.84 vs 211.19 ± 16.13; duodenum: 96.44 ± 9.8 vs 59.24 ± 2.77; jejunum: 87.67 ± 4.9 vs 55.29 ± 3.69; ileum: 94.08 ± 4.25 vs 56.59 ± 1.81; colon: 196.64 ± 20.72 vs 115.69 ± 7.84, respectively, $p<0.05$ for all). Treatment with losartan

successfully prevented this thickness increase in all the portions studied (LM, in μm: stomach: 82.21 ± 9.94; duodenum: 39.95 ± 2.87; jejunum: 37.27 ± 1.97; ileum: 35.85 ± 2.22; colon: 35.92 ± 2.66; CM, in μm: stomach: 251.15 ± 24.28; jejunum: 60.97 ± 4.21; ileum: 63.03 ± 2.00; colon: 123.06 ± 11.84, $p>0.05$ for all compared to CTRL) except in the CM of the duodenum where this increase was only attenuated (73.88 ± 4.06, $p<0.05$ compared to CTRL and STZ). Finerenone treatment did not prevent the reported increase and the results did not differ from those in the CTRL group (LM, in μm: stomach: 105.01 ± 6.14; duodenum: 59.28 ± 3.5; jejunum: 60.4 ± 3.31; ileum: 63.97 ± 6.67; colon: 53.49 ± 3.16; CM, in μm: stomach: 330.07 ± 14.94; duodenum: 101.19 ± 5.8; jejunum: 88.35 ± 3.95; ileum: 94.47 ± 6.9; colon: 206.19 ± 15.18, $p<0.05$ for all compared to CTRL).

The mucosa was increased in STZ animals compared to controls in duodenum (978.47 ± 111.55 μm vs 733.59 ± 67.50 μm), jejunum (678.36 ± 51.51 μm vs 493.57 ± 81.18 μm) and ileum (592.65 ± 26.59 μm vs 395.19 ± 18.02 μm), but none of the treatments employed was able to prevent this alteration.

In general, submucosa presented no differences between all groups, with exception of STZ and STZ+FIN in jejunum where there was an increase in the thickness compared to CTRL group ($p<0.05$).

Representative images of CTRL, STZ, STZ+LOS, and STZ+FIN animals are shown in figure 7, covering all the sections studied. These images offer a detailed visual comparison and the observation of the differences in each analyzed area.

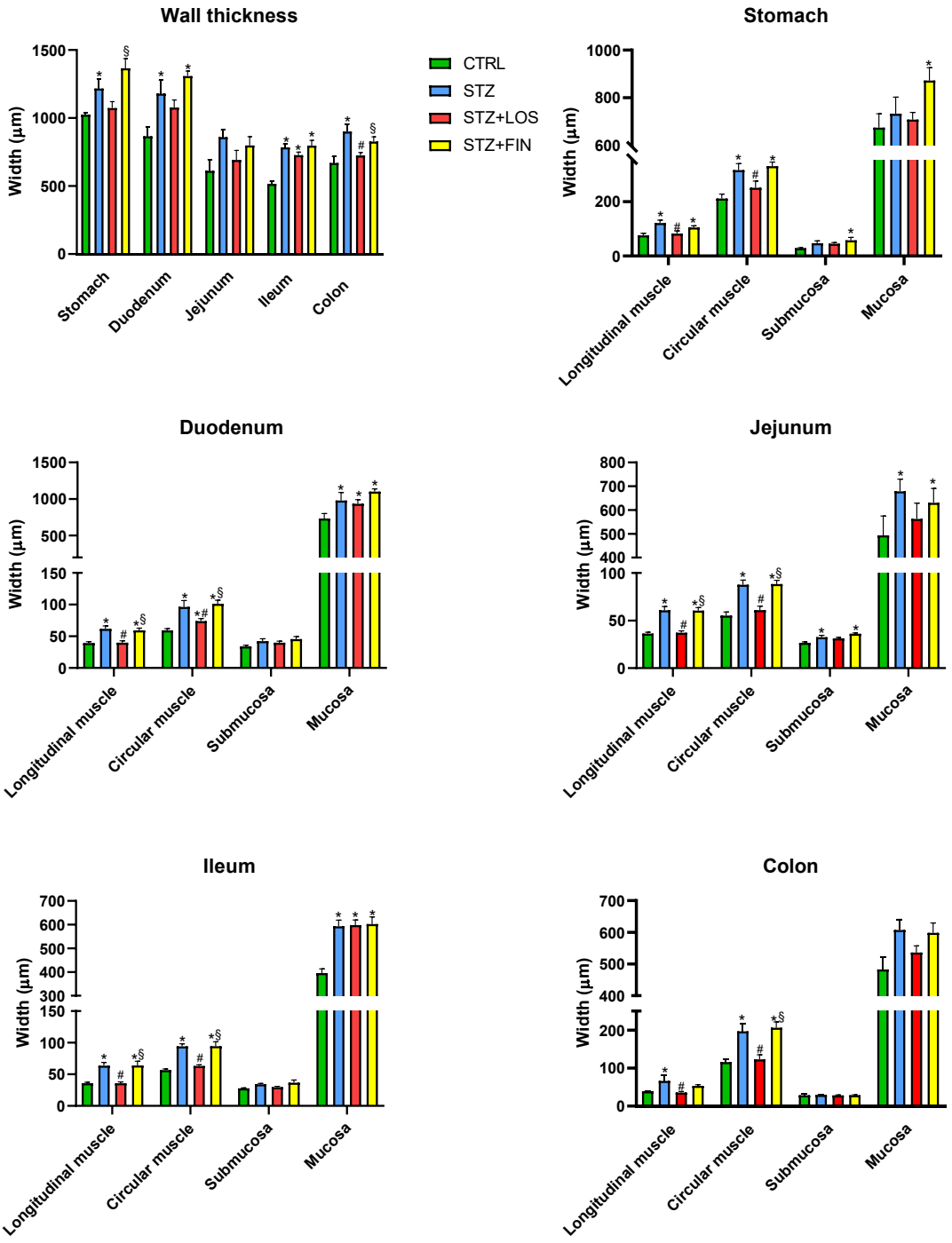

Figure 2 – Microscopic evaluation of the gastrointestinal wall of stomach, duodenum, jejunum, ileum and colon and thickness (µm) of the intestinal layers (longitudinal muscle, circular muscle, submucosa and mucosa) of each intestinal segment of control (CTRL, n = 8), streptozotocin-induced diabetic rats (STZ, n = 8), streptozotocin-induced diabetic rats treated with losartan (STZ+LOS, n=8) and streptozotocin-induced diabetic rats treated with finerenone (STZ+FIN, n=8). Values are mean ± SEM. * Statistical difference ($p < 0.05$) to CTRL; #Statistical difference ($p < 0.05$) to STZ.; §Statistical difference ($p < 0.05$) to STZ+LOS.

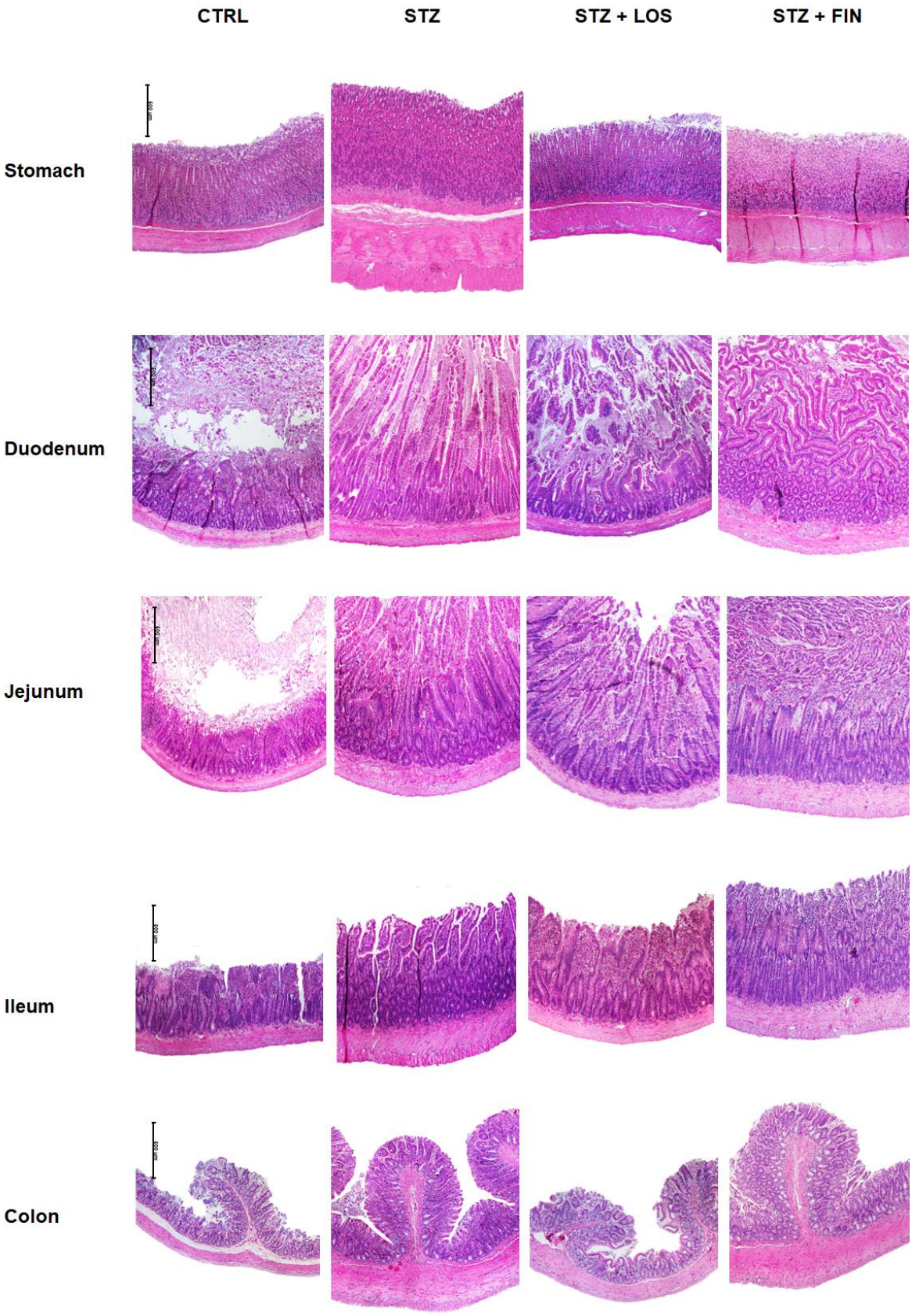

Figure 7 - Representative microscopic photographs of stomach, duodenum, jejunum, ileum and colon of control (CTRL), streptozotocin(STZ)- induced (STZ), STZ treated with losartan (STZ+LOS) and STZ treated with finerenone (STZ+FIN) stained with hematoxylin and eosin, captured using a 40x magnification.

3.4. Total GSH and GSSG Quantification

To investigate the potential causes of the remodeling observed earlier, we measured tGSH and GSSG levels as indicators of oxidative stress, a key factor in the development of diabetic complications. The results of the tGSH quantification revealed an increase in all regions of diabetic animals compared to controls (in nmol tGSH/mg protein: stomach: 17.69 ± 2.05 vs. 6.67 ± 0.88; duodenum: 17.56 ± 0.96 vs. 9.73 ± 1.48; jejunum: 19.96 ± 1.49 vs. 10.11 ± 0.88; ileum: 15.03 ± 1.28 vs. 7.23 ± 0.5; colon: 12.52 ± 1.01 vs. 4.25 ± 0.74, respectively, $p < 0.05$ for all). Treatment with losartan prevented this increase in all regions studied (in nmol tGSH/mg protein, duodenum: 13.57 ± 1.24; jejunum: 9.93 ± 1.53; ileum: 7.79 ± 0.35; colon: 4.75 ± 0.34, $p > 0.05$ compared to CTRL), except in the stomach, where tGSH was lower than in the STZ group but higher than in the CTRL group (12.45 ± 2.64 nmol/mg protein, $p < 0.05$ compared to both CTRL and STZ). In contrast, treatment with finerenone did not affect these alterations in any of the regions studied (in nmol tGSH/mg protein: stomach: 17.97 ± 1.71; duodenum: 17.47 ± 3.02; jejunum: 15.8 ± 0.61; ileum: 18.19 ± 1.24, $p < 0.05$ compared to CTRL), except in the colon, where tGSH levels were decreased compared to STZ but increased compared to CTRL (8.53 ± 0.5 nmol/mg protein, $p < 0.05$ compared to both CTRL and STZ) (figure 8).

Similarly to what was observed with tGSH, GSSG levels were also elevated in all regions of STZ animals compared to controls (in nmol GSSG/mg protein, stomach: 3.03 ± 0.32 vs. 0.59 ± 0.11; duodenum: 2.98 ± 0.60 vs. 0.39 ± 0.07; jejunum: 3.07 ± 0.40 vs. 0.49 ± 0.06; ileum: 2.25 ± 0.24 vs. 0.62 ± 0.09; colon: 1.53 ± 0.24 vs. 0.39 ± 0.09, respectively, $p < 0.05$ for all). Treatment with losartan prevented this increase in all regions studied (in nmol GSSG/mg protein, stomach: 1.07 ± 0.07; duodenum: 1.14 ± 0.07; jejunum: 1.26 ± 0.06; ileum: 0.74 ± 0.15; colon: 0.51 ± 0.10, $p > 0.05$ compared to CTRL). In contrast, treatment with finerenone did not affect these alterations in the stomach (2.55 ± 0.48 nmol/mg protein), duodenum (2.38 ± 0.46 nmol/mg protein) and colon (1.10 ± 0.12 nmol/mg protein) but was able to atenuate GSSG values in the jejunum (1.92 ± 0.22 nmol/mg protein) and ileum (1.16 ± 0.07 nmol/mg protein) ($p<0.05$ compared to both CTRL and STZ) (figure 8).

Regarding the GSH/GSSG ratio, a decrease was observed in all portions of STZ diabetic rats compared to controls (stomach: 5.53 ± 0.90 vs. 12.77 ± 1.52; duodenum: 7.73 ± 1.9 vs. 13.22 ± 1.45; jejunum: 4.93 ± 0.97 vs. 11.65 ± 1.27; ileum: 7.19 ± 0.82 vs. 10.1 ± 1.79; colon: 5.89 ± 0.65 vs. 9.64 ± 0.66, respectively, $p < 0.05$ for all). However, in Losartan treated animals, the ratio was similar to controls in all portions studied (stomach: 9.58 ± 1.98; duodenum: 9.42± 1.11; jejunum: 8.08 ± 1.37; ileum: 11.19 ± 1.09; colon: 10.15 ± 2.36, $p > 0.05$). Finerenone treated animals presented a lower ratio compared to controls in all

portions (stomach: 5.22 ± 1.71; duodenum: 6.00 ± 0.55; jejunum: 4.50 ± 0.63; colon: 4.16 ± 0.49, p<0.05 for all), except ileum (8.11 ± 0.94, p> 0.05) (figure 8).

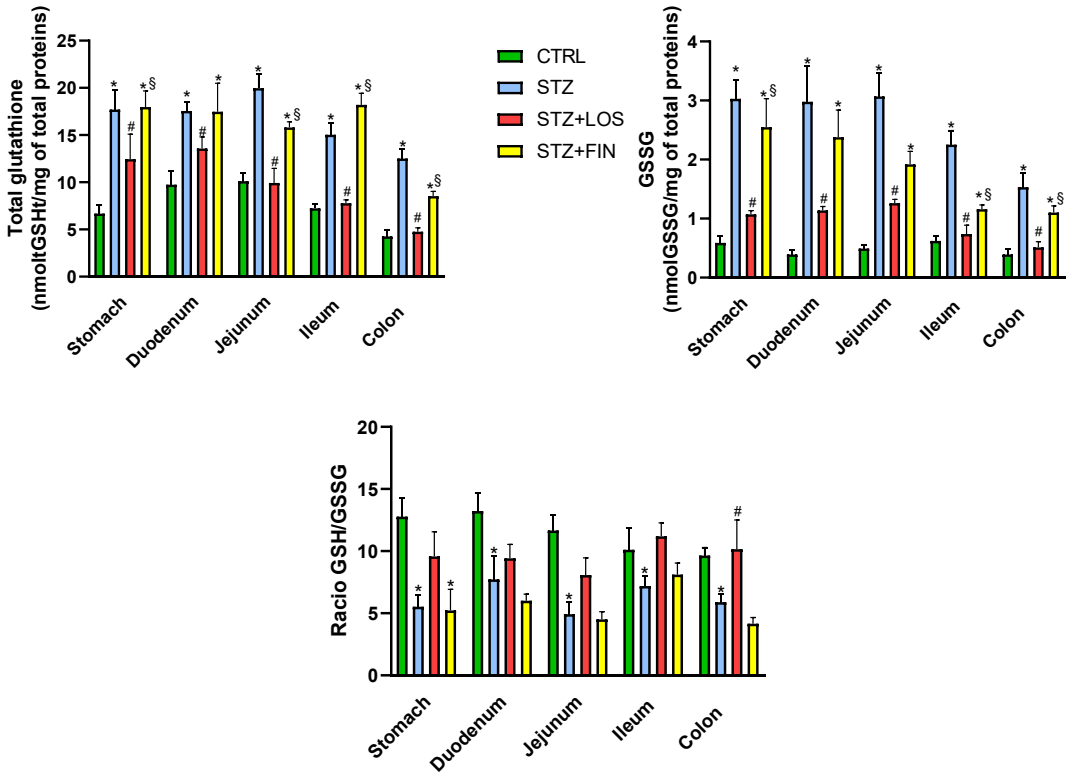

Figure 8 - Glutathione evaluation of gastrointestinal segments (stomach, duodenum, jejunum, ileum and colon) of control rats (CTRL, n = 8), streptozotocin-induced diabetic rats (STZ, n = 8), streptozotocin-induced diabetic rats treated with losartan (STZ-LOS, n=8) and streptozotocin-induced diabetic rats treated with finerenone (STZ-FIN, n=8): total glutathione (tGSH) quantification (nmol GSH/mg protein); oxidized glutathione (GSSG) quantification (nmol GSSG/mg protein) and ratio GSH/GSSG. Values are mean ± SEM. * Statistical difference ($p < 0.05$) to CTRL; #Statistical difference ($p < 0.05$) to STZ.; §Statistical difference ($p < 0.05$) to STZ+LOS.

3.5. ACE and ACE 2 activity

Both ACE Z-FHL and ACE h-HL activities were higher in the serum of all diabetic groups compared to controls (ACE Z-FHL activity in nmol/min, CTRL: 737.7 [605.5, 771.3]; STZ: 1186 [1058, 1398]; STZ+LOS: 1056 [984.1, 1333]; STZ+FIN: 1609 [1007, 1752]; ACE h-HL activity in nmol/min, CTRL: 146 [92.65, 219.5]; STZ: 300.4 [214.4, 365.6]; STZ+LOS: 251.6 [208.1, 393.7]; STZ+FIN: 388.1 [230.7, 471.1], p<0.05 for all). Also, the ACE Z-FHE/h-HL activity ratio was greater than 1 and consistently close to 4, indicating a predominant N-domain activity in all experimental groups (figure 9).

ACE2 activity was higher in the serum of all diabetic groups compared to controls (ACE2 activity in nmol/min, CTRL: 976.4 [733.9, 1469]; STZ: 2537 [1679, 3932]; STZ+LOS: 2345

[1422, 3019]; STZ+FIN: 2486 [1527, 3374], p<0.05 for all). Also, the ACE2/ ACE Z-FHE activity ratio was greater than 1 in all experimental groups (figure 9).

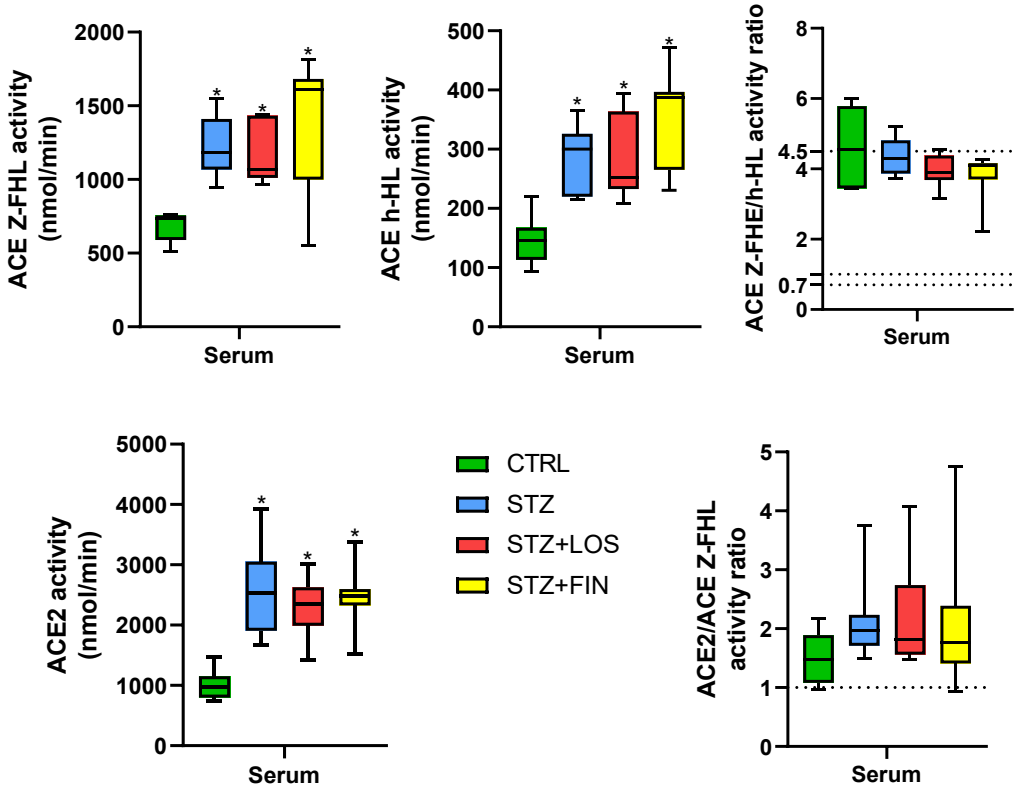

Figure 9 – Angiotensin Converting Enzyme (ACE) catalytic activities, including Z-FHL and h-HL substrates, as well as the ACE Z-FHL/h-HL ratio and the ACE2/ACE activity ratio in the serum of control rats (CTRL, n = 8), streptozotocin-induced diabetic rats (STZ, n = 8), and streptozotocin-induced diabetic rats treated with losartan (STZ+LOS, n = 8) or finerenone (STZ+FIN, n = 8). Values are median [95% confidence limits]. * Statistical difference to CTRL (p < 0.05)

ACE Z-FHL activity was higher in the intestinal portions of STZ diabetic animals compared to controls across all studied sections (in nmol/min/mg of total proteins, stomach: 32.22 [12.85, 82.47] vs 6.77 [0.95, 19.79]; duodenum: 29.9 [25.01, 99.15] vs 18.76 [2.14, 26.21]; jejunum: 46.49 [23.42, 86.52] vs 24.91 [3.03, 35.45]; ileum: 21.51 [11.18, 58.15] vs 6.31 [3.07, 9.67]; colon: 15.13 [6.98, 45.97] vs. 5.07 [2.46, 7.93], respectively, p < 0.05 for all). Treatment with losartan did not affect this increase, as the intestinal portions of these animals exhibited ACE Z-FHL activity similar to that of STZ animals and statistically different from the controls (in nmol/min/mg of total proteins, stomach: 25.26 [11.79, 112.8]; duodenum: 47.82 [18.02, 90.58]; jejunum: 50.54 [30.37, 68.61]; ileum: 24.52 [10.04, 90.00]; colon: 24.32 [2.53, 64.59], p < 0.05 for all). In finerenone-treated animals, the ACE-Z-FHL

activity in the ileum was higher than in controls (17.57 [6.25; 89.9] nmol/min/mg of total proteins) differed from controls ($p < 0.05$), while the remaining portions showed no significant difference compared to any of the other experimental groups (figure 9).

Looking at ACE h-HL activity, it was higher in the intestinal portions of STZ and LOS-treated animals compared to controls in the stomach (in nmol/min/mg of total proteins, STZ: 7.53 [3.58, 30.71] and STZ+LOS: 7.19 [2.97, 12.75] vs. CTRL: 0.86 [0.15, 4.8], $p < 0.05$), duodenum in nmol/min/mg of total proteins, STZ: 14.27 [2.60, 35.21] and STZ+LOS: 20.71 [11.52, 44.51] vs. CTRL: 2.03 [0.11, 11.15], $p < 0.05$), and jejunum (in nmol/min/mg of total proteins, STZ: 13.87 [11.55, 39.64] and STZ+LOS: 19.47 [10.56, 31.78] vs. CTRL: 3.63 [0.24, 8.59], $p < 0.05$).

The ACE Z-FHE/h-HL activity ratio was greater than 1 in all experimental groups and higher than 4.5 in most of the portions, indicating a N-domain predominant activity. The results were similar between the four experimental groups studied, except for the duodenum and jejunum of STZ and LOS-treated animals, which showed a decreased ratio compared to controls (duodenum, STZ: 2.11 [1.39, 7.63] and STZ+LOS: 1.39 [0.56, 7.87] vs. CTRL: 9.37 [1.84; 24.83]; jejunum, STZ: 3.48 [1.29, 6.45] and STZ+LOS: 2.19 [0.96, 5.35] vs. CTRL: 8.03 [4.79; 13.80], $p < 0.05$) (figure 9).

Contrary to what was observed in the serum, ACE2 activity was similar across all experimental groups, including diabetic animals, except in the jejunum and ileum of losartan treated diabetic animals. In these sections, ACE2 activity was higher compared to controls (in nmol/min/mg of total proteins, jejunum: 146.3 [103.1, 205.7] vs. 114.1 [27.94, 163.3]; ileum: 96.98 [70.92, 191.00] vs. 25.25 [17.50, 65.70], respectively, $p < 0.05$) (figure 10).

The ACE2/ ACE activity ratio was close to 1 in the control groups and was decreased in all diabetic portions compared to controls (stomach: 0.07 [0.02, 0.86] vs. 1.06 [0.51, 5.00]; duodenum: 0.07 [0.01, 0.19] vs. 1.30 [0.54, 3.88]; jejunum: 0.16 [0.07, 1.19] vs 1.30 [0.55, 2.91]; colon: 0.06 [0.02, 0.47] vs. 0.34 [0.13, 1.82], $p< 0.05$ for all), except ileum ($p>0.05$). Losartan-treated animals also exhibited this decrease in the stomach (0.09 [0.06, 0.36] and duodenum (0.1 [0.02, 0.32]), while only the stomach of finerenone-treated animals (0.11 [0.04, 0.39]) showed the same pattern ($p < 0.05$ for all) (figure 10).

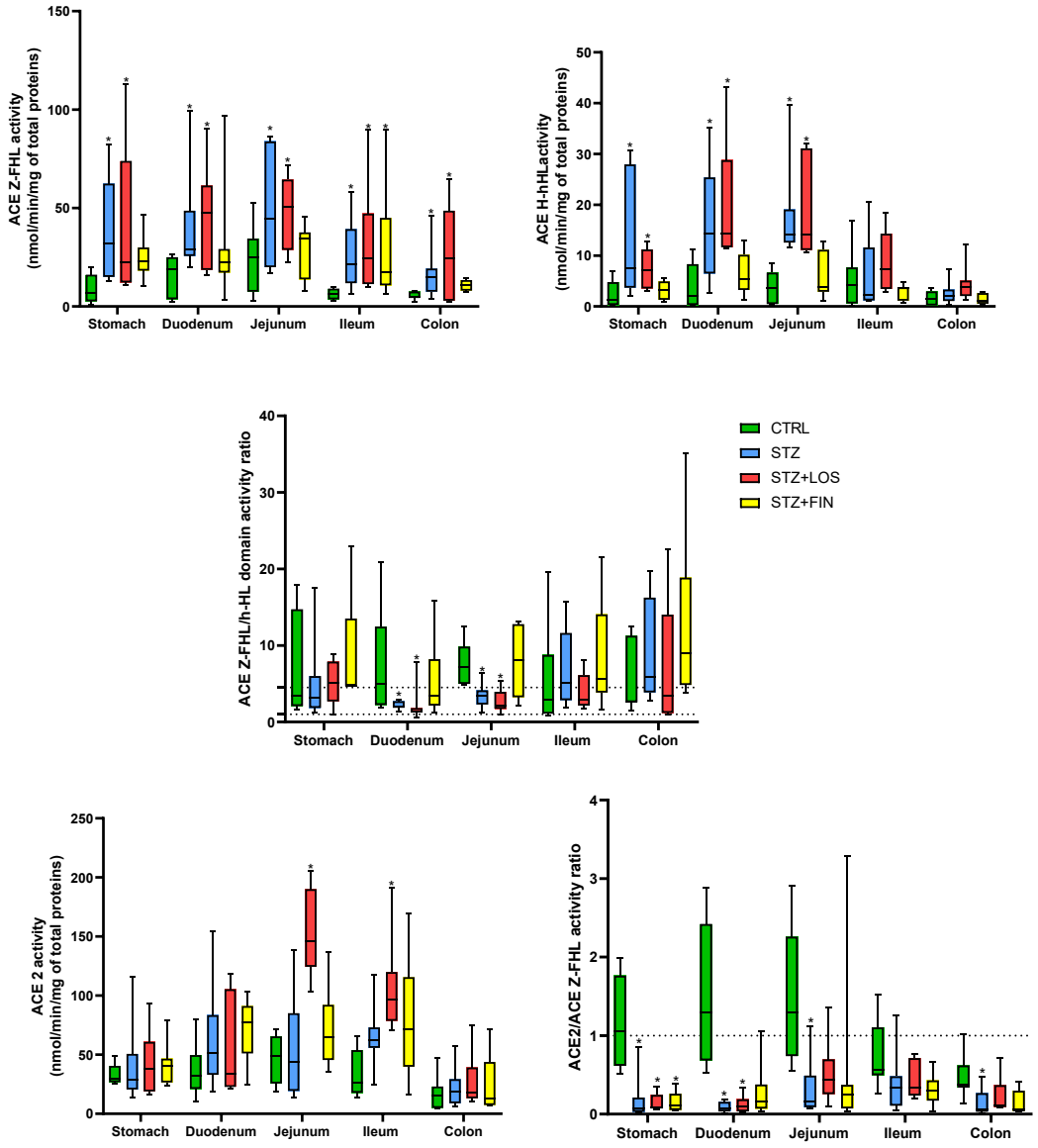

Figure 10 - Angiotensin Converting Enzyme (ACE) catalytic activities, including Z-FHL and h-HL substrates, as well as the ACE Z-FHL/h-HL ratio and the ACE2/ACE activity ratio in the gastrointestinal tract (stomach, duodenum, jejunum, ileum and colon) of control rats (CTRL, n = 8), streptozotocin-induced diabetic rats (STZ, n = 8), and streptozotocin-induced diabetic rats treated with losartan (STZ+LOS, n = 8) or finerenone (STZ+FIN, n = 8). Values are median [95% confidence limits]. * Statistical difference ($p < 0.05$)

4. Discussion

Diabetes is known to induce significant alterations in the GI tract, as was already described by our and other groups[10,24,35], that may contribute to complications associated with the disease and decrease quality of life of diabetic patients[10]. Our study is the first to investigate the effects of losartan and finerenone on the GI tract of STZ-induced diabetic animals,

providing novel insights through histopathological analysis, redox status evaluation, and the assessment of both systemic and local RAAS. Notably, the prevention of muscular hypertrophy and oxidative stress by losartan observed in this study highlights the critical role of AT_1 receptor-mediated mechanisms in these pathological changes, offering new avenues for therapeutic intervention.

As expected, the STZ-induced diabetic rats showed typical diabetes signs, including hyperglycemia, polyphagia, polydipsia, and weight loss[43]. These symptoms arise primarily due to a deficiency in insulin production due to selective destruction of pancreatic β-cells[44], leading to elevated blood glucose levels that disrupt normal metabolic functions[5]. The observed hyperglycemia triggers compensatory mechanisms such as polydipsia[45], polyphagia[46] and weight loss that was more pronounced on the second day as already described by our [24] and other studies[47]. In this context, losartan and finerenone have been explored for their potential in treating diabetes related complications[48,49], but their effects on the core diabetic symptoms remain under investigation as these medications do not appear to directly influence the classic symptoms of diabetes. Losartan may offer mild improvements in insulin sensitivity, but its action does not significantly lower blood glucose levels or alleviate the compensatory mechanisms driven by hyperglycemia[50]. Similarly, finerenone's role in managing renal complications in diabetes does not extend to improving glucose metabolism or reducing the symptoms associated with metabolic dysregulation[51]. Therefore, it is not unexpected that no significant differences were observed in the typical diabetic signs between the STZ group and the two treated groups.

The increased colon length and intestinal perimeter was already described before [24,52]. In the context of the polyphagia observed in diabetic animals, it is noteworthy that intestinal smooth muscle cells (SMC) exhibit remarkable plasticity, adapting to functional demands through remodeling[52]. Jervis and colleagues[53] proposed that the enlargement of the intestine in diabetic animals may therefore be an adaptive response to polyphagia. In a study with diabetic rats induced by alloxan, these authors also described an enlargement of the diameter and length of small intestine and colon[53]. Curiously, other causes of polyphagia like lactation[54] or hypothalamic lesions[55] may also induce GI enlargement - similar to the one seen in diabetes, reinforcing this theory. However, this hypothesis was contradicted by another study that showed that when the food intake of diabetic rats was matched to that of controls (avoiding polyphagia), the intestinal mass of diabetic animals continued to be higher[56]. Therefore, it seems prudent to assume that only part of the intestinal growth will depend on higher food consumption.

Histopathological changes align with macroscopic observations and can be categorized as mucosal and muscular growth. Mucosal growth in diabetic animals may be partially driven by polyphagia, mediated by glucagon-like peptide 2 (GLP-2), which promotes epithelial

proliferation and intestinal growth[57]. The mucosal thickening also appears to be involved with a suppression of apoptosis that happens in the first week after STZ injection, returning to normal 3 weeks after, suggesting a transient effect[58]. AGE and RAGE accumulation in the mucosa may also be related to increased mucosa thickness[59], besides affecting digestive function by changing the properties of the intestinal epithelial cells and digestive enzymes activity[60].

Regarding the muscular layers of the GI wall, previous work demonstrated an extensive remodeling of the diabetic GI tract associated with an increased production and accumulation of collagen type I and AGE/RAGE[10]. The collagen fibers accumulate predominantly around and between SMCs, contributing to the thickening and stiffening of the gastrointestinal wall. This accumulation leads to a reduction in the gut's resting compliance, impairing its ability to expand and contract properly[56,61]. The increased collagen deposition disrupts normal tissue architecture, making the gut less flexible and more resistant to normal physiological movements, which can exacerbate gastrointestinal complications commonly observed in diabetic conditions[10]. In addition to extracellular matrix remodeling, authors also found SMC hypertrophy, with increased number of contractile protein actin and myosin[61]. Increased production of AGE and their receptors was also found in ileum muscular layers, an observation that was related to its increase in thickness[59].

This is the first study to demonstrate that losartan treatment effectively prevents intestinal distension and the hypertrophy of the muscular layers in the GI wall, highlighting Ang II as a crucial, previously underappreciated player in the gastrointestinal complications of diabetes. Ang II, via the activation of AT_1 receptors, plays a pivotal role in the structural alterations seen in various organs, including the cardiovascular and renal systems in diabetes[62,63]. Previous studies have shown that blocking AT_1 receptors reduces fibrosis and muscle hypertrophy in diabetic animal models of cardiovascular disease[26]. Locally, Ang II binding to AT_1 receptors activates multiple intracellular signaling pathways that stimulate profibrotic downstream effects, namely cellular proliferation and accumulation of ECM[64]. This overactivation of the RAAS in diabetic patients is primarily driven by hyperglycemia, which directly and indirectly stimulates the production of Ang II[65]. The prevention of muscular hypertrophy by losartan supports the role of RAAS in these pathological changes as blocking AT_1 receptors may mitigate these effects in the gut, preserving normal muscle architecture and function. However, the increased mucosal thickness was not prevented by losartan treatment. A study using enalapril concluded that ACE inhibition alleviated both morphological and functional changes in the diabetic mucosa[66]. While losartan did not prevent the increased thickness, it remains possible that other underlying effects may have occurred, which are yet to be fully understood.

In contrast, finerenone did not prevent the increased muscle thickness. This indicates that the observed remodeling may be more dependent on Ang II signaling than on aldosterone-mediated pathways. While mineralocorticoid receptor antagonists have been shown to reduce fibrosis and inflammation in other tissues, such as the kidney and heart[49,51], their limited effect on the GI tract suggests a distinct regulatory mechanism that is more effectively targeted by AT_1 receptor antagonism.

Regarding oxidative stress, ROS are known to be key mediators of fibrosis induced by Ang II[67,68] and have been associated to the GI damage caused by diabetes[34]. To assess the redox status in the GI tract of diabetic animals, we measured tGSH and the GSH/GSSG ratio - key indicators of oxidative stress[69]. GSH, the primary intracellular antioxidant, plays a crucial role in signaling, detoxification, and reactive species inactivation[70]. We also measured GSSG, which increases following oxidative stress[30]. This approach was chosen over other markers like protein carbonylation or lipid peroxidation, as those reflect mostly end-stage damage[71]. Given our short-term diabetes model, we aimed to capture early redox alterations and potential intercellular mechanisms. In these study, diabetic animals exhibited increased local levels of tGSH, GSSG and a decreased GSH/GSSG ratio, indicating an imbalance in redox homeostasis and heightened oxidative stress in the GI tract. Only one previous study has examined local GSH levels in the GI tract in a T2D model, reporting a decrease in both tGSH and the GSH/GSSG ratio[35]. Although our findings may seem contradictory, previous studies have shown that, systemically in diabetes, total GSH levels initially increase due to the redirection of precursors to counteract oxidative stress[72]. This leads to a higher conversion of GSH to GSSG, ultimately reducing the GSH/GSSG ratio[73]. However, in chronic diabetes, the depletion of GSH precursors results in lower total GSH levels[74], while the remaining glutathione continues to be oxidized into GSSG. Consequently, both tGSH and the GSH/GSSG ratio decrease over time[75]. Given that this study focuses on short-term diabetes, it is not surprising that we observed elevated tGSH levels and a reduced GSH/GSSG ratio, driven by the higher GSSG levels in the STZ groups compared to controls. Increased levels of GSSG in diabetes were previously described and are related to increased utilization due to increased levels of ROS[76,77]. The decreased GSH/GSSG ratio, which is approximately half in these diabetic samples compared to controls, aligns with findings previously reported by our group[35] and the plasmatic values described by others[77,78].

Losartan treatment prevented these changes, while finerenone had no significant effect. This finding is consistent with reports that Ang II exacerbates oxidative stress by increasing ROS production and decreasing GSH[79]. To date, there are no other studies specifically examining the effects of losartan on the GI tract in diabetic animal models. However, research in diabetic animals has demonstrated that AT_1 receptor antagonists, such as

losartan and valsartan, even at moderate doses, can have a profound and rapid effect in suppressing systemic ROS[80,81]. This suppression leads to a reduction in oxidative stress and related cellular damage, which are common complications of diabetes[80]. These findings highlight the potential therapeutic benefits of using these medications not only for managing blood pressure but also for mitigating oxidative damage in diabetic conditions[80,81]. The decreased production ROS in the gut following losartan administration explains why treated animals did not exhibit increased levels of tGSH or a reduced GSH/GSSG ratio, like the STZ group. On the other hand, the lack of effect of finerenone suggests that aldosterone signaling may not be the primary driver of oxidative stress in the diabetic GI tract. Previous studies have indicated that aldosterone contributes to oxidative damage mainly through upregulation of ROS production in cardiovascular and other tissues[25,62], but its role in gut pathology remains less clear. This may explain why MR blockade alone was insufficient to restore redox balance in the present study.

In this study, we assessed ACE activity both locally in the GI tract and in circulation. Systemically, all diabetic animals, irrespective of treatment, exhibited increased ACE and ACE2 activity, confirming previous reports that diabetes is associated with dysregulation of the RAAS[82,83]. However, the fact that the ratios of ACE activity (Z-FHL/hHL) and the ACE2/ACE ratio remained consistent across all experimental groups suggests that the N and C-domain activities are increased in proportion to each other. The same trend is observed when examining ACE2 and ACE. This balance was already previously described, and the systemic upregulation of ACE2 suggests a compensatory mechanism aimed at counteracting the deleterious effects of heightened ACE activity and Ang II production[84].

This is the first study to investigate local ACE and ACE2 activity and the Z-FHL/h-HL and ACE2/ACE ratios in the diabetic GI tract, and we found differences between systemic and local RAAS activity. While systemic ACE and ACE2 were both upregulated, local ACE2 activity in the GI tract remained unchanged, and local ACE activity was significantly increased. This discrepancy between systemic and local RAAS activity aligns with previous findings suggesting that tissue-specific RAAS alterations may contribute differently to diabetic complications[62,63]. This study utilized both Z-FHL and h-HL to measure ACE activity in various tissues, highlighting how the Z-FHL/h-HL ratio can provide insights into the functional balance between the N- and C-domains[85]. Some diseases have shown to increase or decrease this ratio[85,86]. Research on the kidney of diabetic rats has shown that alterations in ACE activity, particularly in the N-domain, might contribute to tissue-specific changes in ACE activity[87]. However, this contradicts our observations in the diabetic gut, where we noted a decrease in the Z-FHL/h-HL ratio in certain diabetic tissues. This suggests a possible shift in ACE activity, with increased C-domain activity - the domain primarily responsible for converting Ang I into Ang II. This increased local ACE activity in

diabetic animals further supports the notion that Ang II is a key mediator of GI tract remodelling. Since losartan effectively prevented changes in muscular thickness and oxidative stress, it is plausible that blocking the effects of Ang II at the tissue level is essential for mitigating these alterations. On the other hand, the lack of significant change in ACE2 activity locally resulting in a decrease ACE2/ACE ratio suggests that alternative pathways, independent of the ACE2/Ang-(1-7)/Mas axis, may be at play in the GI tract of diabetic animals. Additionally, the ACE2/ACE ratio was reduced in diabetic tissues, further indicating an imbalance in the RAAS components, which favours increased Ang II production. This imbalance, not compensated by enhanced degradation, could contribute to tissue-specific pathologies. In fact, an increase in ACE activity, not accompanied by a corresponding rise in ACE2 locally, was also observed in the diabetic kidney and is considered a key driver of renal injury[84,88].

Given that losartan's mechanism of action focuses on preventing the binding of Ang II to its receptor, one might question the rationale for studying ACE activity in losartan-treated animals and whether any effect is to be expected. Interestingly, losartan has been shown to bind to a specific pocket on the ACE enzyme without inhibiting its catalytic activity[89]. This interaction suggested that losartan could modulate ACE function through allosteric mechanisms, potentially influencing the enzyme's interaction with other substrates or proteins without directly affecting its enzymatic activity[89]. However, in this study, neither losartan nor finerenone had any effect on ACE or ACE2 activity in both serum and the gastrointestinal tract. Although losartan has the ability to interact with ACE, the potential influence of this interaction remains to be uncovered.

5.Conclusion

Our findings highlight the differential effects of losartan and finerenone on diabetes-induced alterations in the GI tract. The prevention of muscular hypertrophy and oxidative stress by losartan underscores the importance of AT_1 receptor-mediated mechanisms in these pathological changes. In contrast, the lack of effect of finerenone suggests that MR blockade alone is insufficient to counteract the remodeling and oxidative imbalances observed. Additionally, the observed discrepancies between systemic and local RAAS activity further emphasize the complexity of RAAS regulation in diabetes.

These results suggest that ARBs, such as losartan, may offer therapeutic benefits in preventing diabetes-induced GI tract remodeling, whereas MRAs do not provide the same level of protection. Future studies should investigate the precise mechanisms governing tissue-specific RAAS regulation and assess the potential of ARBs not only as a preventive strategy, as observed in this study, but also as a possible therapeutic intervention.

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Chapter 3 – Domestic pets results

3.1. Ultrasonographic evaluation of the normal gastrointestinal wall in dogs and cats: a systematic review on study design and imaging outcomes

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Abstract

Diagnostic ultrasound (US) is a non-invasive, cost-effective imaging modality widely used for evaluating the gastrointestinal (GI) tract in companion animals. It provides critical information on wall thickness and layer differentiation, aiding in the assessment of both normal and pathological conditions. Despite its diagnostic relevance, standardized ultrasonographic reference values for the GI tract in dogs and cats remain inconsistent across studies. This study aimed to systematically review the quantitative ultrasonographic characteristics of the normal GI tract wall in dogs and cats and compile a consensus-based reference table for overall wall thickness and individual layer proportions to enhance clinical interpretation. A systematic literature search was conducted in PubMed and Scopus to identify studies assessing the ultrasonographic features of normal GI segments, from the stomach to the colon, in healthy dogs and cats. Twelve studies met the inclusion criteria, with six focused on dogs and six on cats. Findings confirmed the existence of reference values for GI wall thickness and its layers in both species. However, discrepancies were noted in weight-based classifications for dogs, and the stomach of adult dogs remains poorly studied. Additionally, in this species, the evaluation of specific gastric structures, such as rugal folds and inter-rugal spaces, is underexplored. Ultrasound serves as a valuable tool for GI assessment in veterinary medicine, yet the dispersion of reference values across multiple studies may hinder accessibility. Establishing standardized ultrasonographic parameters could improve diagnostic accuracy and clinical decision-making.

1.Introduction

Diagnostic ultrasound (US) is a non-invasive, cost-effective, readily accessible medical imaging technique that utilizes high-frequency sound waves to generate real-time images of the body[1]. This versatile imaging modality plays a pivotal role in clinical practice, offering valuable diagnostic insights across a broad spectrum of medical applications[2]. Among its many uses, ultrasound has proven to be an essential tool in the assessment, diagnosis, treatment, and ongoing management of various medical conditions, including those affecting the gastrointestinal tract (GIT)[3].

Within the GI system, ultrasound provides a wealth of information that is critical for evaluating both normal and pathological conditions. This includes assessing wall thickness and its individual layers, characterizing luminal contents, evaluating motility, and visualizing adjacent organs and structures such as the stomach, pancreas, mesentery, lymph nodes, and peritoneum[1,4]. A systematic approach to scanning the GIT ensures a comprehensive evaluation, with key segments routinely examined including the stomach, pyloroduodenal junction, duodenum, jejunum, ileum, ileocecocolic junction, and colon[4].

A thorough understanding of both normal and abnormal ultrasonographic presentations of the GIT in companion animals, such as dogs and cats, significantly enhances diagnostic accuracy when investigating gastrointestinal disorders[3]. The most common diseases or conditions that may affect the normal thickness and/or layering of the GIT wall include inflammation, chronic enteropathies, neoplastic and non-neoplastic disorders, ulceration, and rupture[5]. These alterations, commonly seen in both dogs and cats, often lead to changes in echogenicity and/or thickness of the intestinal wall and may selectively involve some intestinal layers[6–9].

Given the diagnostic significance of intestinal wall thickness and its layered structure, it is imperative to establish robust and standardized reference values for the ultrasonographic appearance of the entire GIT. This includes defining normal full-thickness measurements and quantitatively characterizing the proportions of each layer[4]. Standardized imaging protocols and validated reference values facilitate objective assessments, improve diagnostic consistency, and enhance the ability to detect and monitor disease progression. By refining these parameters, veterinary professionals can optimize the use of ultrasound in the evaluation of gastrointestinal health and disease, ultimately leading to more accurate diagnoses and improved patient outcomes.

We hypothesized that miscellaneous material and methods may influence consensual and objective imaging outcomes. Accordingly, the aims of this study were to: 1) provide a contemporary systematic review of the qualitative and quantitative ultrasonographic features of the normal GIT wall in dogs and cats; 2) develop a collective imaging feature-based table with consensus intervals for the overall wall thickness and corresponding

individual layers of the GIT in dogs and cats, to facilitate US features of the GIT interpretation in clinical practice and 3) to identify existing knowledge gaps in the US features of the normal GIT in companion animals.

2. Material and Methods

2.1. Literature search and search terms

The Pubmed and Scopus databases were searched from their inception until the most recent search on [17/02/2025], to identify all articles related to US of the GIT in dogs and cats. The search terms used in the databases included: [ultrasound OR sonography OR ultrasonography (MeSH terms)] AND [gastrointestinal tract (Mesh terms) OR intestinal OR colon (MeSH terms) OR stomach (MeSH terms)] AND [dog OR cat (MeSH terms) OR canine OR feline OR pets (MeSH terms)].

2.2. Inclusion and exclusion criteria

According to the inclusion criteria, studies will be eligible if: i) are published in English or Portuguese; ii) have full text availability; iii) are performed in healthy canine and feline patients without any history of GI disease; iv) evaluate any segment of the GIT, from stomach to colon, via abdominal ultrasound and v) address normal features of GIT in ultrasound, including wall thickness. Regarding the exclusion criteria, studies will be rejected if they are: i) reviews or systematic reviews; ii) performed in humans, laboratory animals or other animal species. Finally, to ensure that no relevant articles were unidentified, the reference lists of all the selected articles will be scrutinized.

2.3. Study selection

All studies identified in the search will be assessed by title and abstract by two authors working independently [MEM and CSB] and after the individual selection, disagreements will be solved by consensus or through the final decision of a third author (MDA). Those that are irrelevant based on title, abstract or study type [case reports, letters to the editor, comments, or review articles] will be excluded in this phase of initial screening. The remaining articles will be evaluated by their full text for their appropriateness to the inclusion criteria by the same two authors. Once again, in case of disagreement, a third author had the final decision (MDA).

2.4. Data review process and analysis of studies

For each study the following data will be collected: i) study population (species, gender, age, weight, number of patients, assessment of normal status, alive/cadaver); ii) study design (type of study, animal awake/sedated/anesthetized, degree of stomach distension, full thickness/individual layering measurements, probe frequency, imaging planes, variables assessed, present/absent histopathologic evaluation); iii) imaging outcomes, iv) conclusions and v) limitations of the study.

The design and writing of this review will be based on the guidelines recommended in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement.

3. Results

A total of 1708 results were identified through searches in the PubMed and Scopus databases, and their respective titles and abstracts were carefully reviewed. After applying inclusion and exclusion criteria, 1546 studies were deemed ineligible, leaving 162 for a comprehensive full-text assessment. Out of these, 102 articles were excluded for not meeting the inclusion criteria. The remaining articles were categorized into two groups: those related to the evaluation of normal gastrointestinal ultrasound and those concerning pathological evaluation. Among these, 11 articles fulfilled all the inclusion criteria for this paper. Additionally, one relevant manuscript was discovered by consulting the reference lists of the initially selected articles. Consequently, a total of 12 articles were included in the final review (see Figure 1), and data from these articles were collected and summarized (Table 1 and 2).

3.1. Study population

Out of the 12 articles that examined the typical US appearance of the GIT in small animals, six were dedicated to cats (using 9 to 38 cats)[10–15], and the remaining six focused on dogs, ranging the number of animals examined from 12 to 231[16–21]. In most of these articles, males were either the predominant subjects or their gender was not specified[10,12,17,19,21]. Among the papers discussing cats, they predominantly studied young adults to adults as subjects, while two of the papers discussing dogs were focused on puppies[16,17], one on puppies and adults[21], with the remainder dedicated to young adults to adults. Two papers did not specify an age group at all[15,19], and other two only assumed a possible age based on dentition[13,18]. None of the papers investigated the GIT of older animals. One of the cat-focused papers did not mention the weight of the subjects[10], while other specified a “normal body condition”[15].The weights of the remaining cats ranged from 3.6 to 5.2 kg; however, the

results were not categorized based on different weight ranges. Only one of the dog papers did not specify the weight of the animals, but they were divided into groups according to the weight[20]. The papers hat studied puppies evaluated the GIT according to age and weight[16,17,21]. Three papers categorized the results based on different weight intervals[19–21], while the last did not categorized by weight since the focus was to correlate with histopathology findings [18].

All dogs were alive and healthy when examined, except in one case where the animals were euthanized for reasons unrelated to gastrointestinal disease[18]. All cats were also alive during the ultrasound except for one study where they used cadavers euthanized for reasons unrelated to gastrointestinal tract disorders, such as behavioral or orthopedic problems[13]. In another paper, the cats were euthanized immediately after imaging[15]. Most of the authors considered the absence of GI disease based on physical exams and clinical history. Only two papers did not describe any assessment of normal GIT status[15,18].

3.2. Study design

All studies are observational. All dogs were awake during examination and were physically restrained (except the ones that were euthanized[18]). Regarding cats, only one study kept the animals awake and restrained manually during the examination[11], while the remaining authors either sedated the cats or put them under general anesthesia.

Only two studies measured the degree of distension of the stomach[10,21] and histopathology was performed in six studies[10,13–15,18,21]. The frequency of the probe was not specified in one of the studies[20] and varied significantly among the remaining papers, ranging from 5 to 18 MHz. Higher probe frequencies were generally used in cat studies. The imaging plane also varied among the studies, with the transverse plane present in all except one[14].

In the cat studies, several variables were measured, including: 1) full thickness of stomach[10,12,15]; 2) thickness of the individual layers of the stomach wall[12]; 3) full thickness of small intestine[10–13,15]; 4) thickness of the individual layers of the small intestine wall[11–13]; 5) ileocolic region[15]; 6) cecum thickness and layering[14]; 7) full thickness of colon[10,12,14,15]; 8) thickness of the individual layers of colonic wall[12]. In three studies, the variables measured were full thickness of stomach, small bowel and colon[16,17,21] and thickness of the individual layers in the same portions[17], correlating with age and body weight. The ultrasound image of the wall layering was also described[16,21].

In the adult dog studies, the variables measured were: 1) full thickness of the small intestine[18–20]; 2) thickness of the individual wall layers of the small intestine[18,20]; 3) full thickness and individual layers of colonic wall[20].

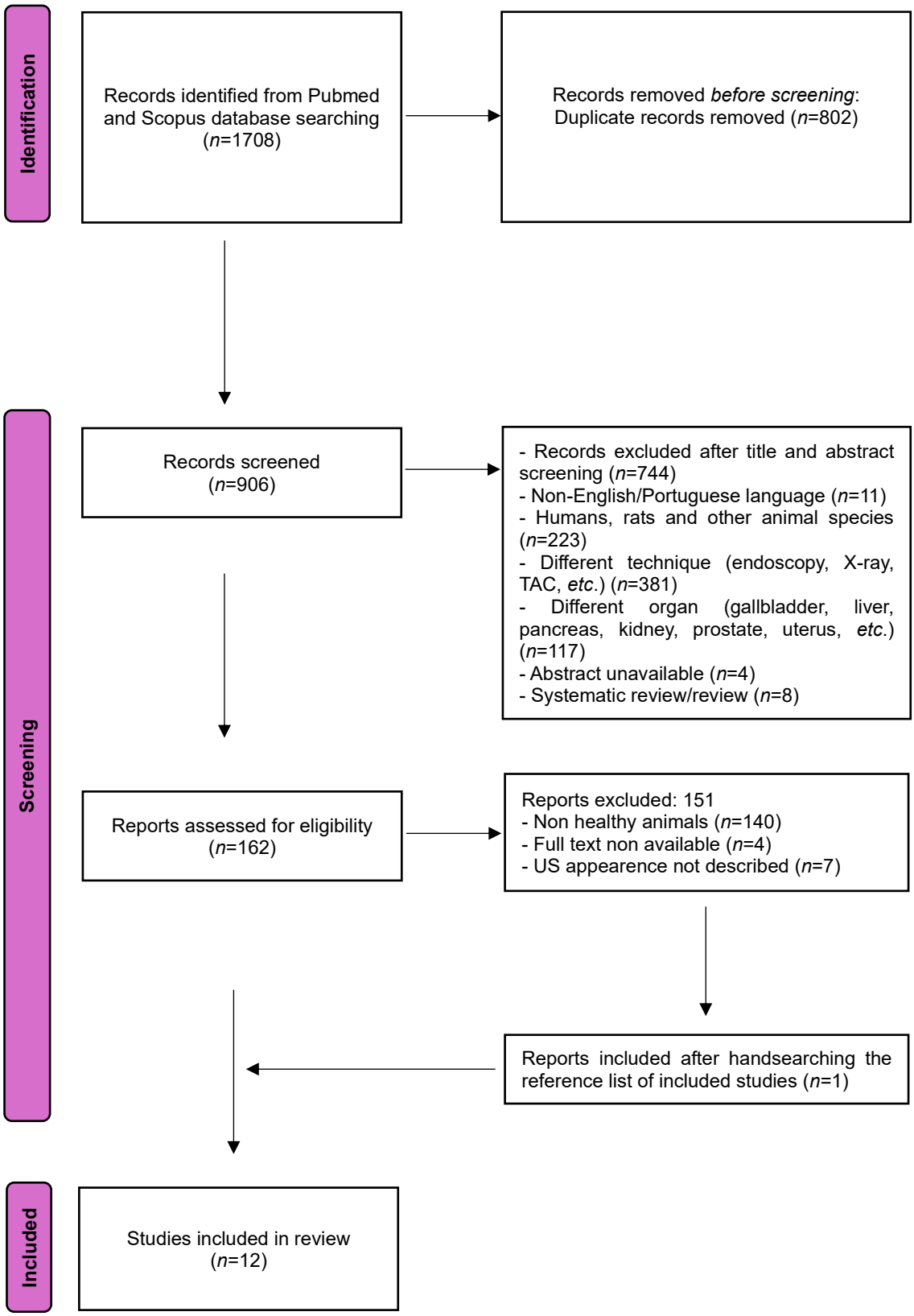

Figure 1. PRISMA flowchart of selected studies.

Table 1. Analysis of the publications regarding US evaluation of the normal GIT in cats.

Study	Animals	Variables / measurements	Normal GI status	Conclusions	Limitations of the study
Newell *et al.*, 1999	*n*=14 1-9 years No gender No weight Alive	**1.** Full wall thickness. **2.** Stomach (rugal, inter-rugal), proximal duodenum, small intestine, descending colon. **3.** Three levels of stomach distension. **4.** Awake and sedated (ketamine hydrochloride + acepromazine maleate). **5.** Three measurements *per* anatomic region (average). **6.** Sagittal and transverse plans. **7.** 10 MHz Probe.	Histopathology Physical and laboratory evaluation (CBC, serum chemistry, urianalysis, fecal examination)	**1.** Sedation increases full thickness of the duodenum as a single factor but did not significantly affect any of the parameters measured. **2.** The thickness of the rugal folds was significantly higher than the thickness of the inter-rugal regions, meaning that separate standards of normal thickness should be recognized for these two functional areas of the stomach. **3.** Distension of the stomach does not significantly change the thickness of the rugal folds or inter-rugal regions. **4.** Reports full wall normal thickness of stomach (rugal and inter-rugal foals), proximal duodenum, small intestine, descending colon.	**1.** Two cats (2/14) had mild histologic evidence of colitis with variable inflammatory cells, although there was no destruction or alteration of the normal histologic architecture of the colon (not known if these cats presented larger measurements for the colon). **2.** Not all variables were assessed for each cat, not allowing individual variations according to distension and sedation status. **3.** No differentiation of gastric segments (fundus, pylorus), neither jejunum nor ileum (overall small bowel). **4.** Limited statistical value (relative low numbers). **5.** Unknown gender.
Goggin *et al.*, 2000	*n*=11, initially *n*=9, after histopathology No age 4 females Normal body condition Alive	**1.** Full wall thickness. **2.** Gastric fundus, pylorus, duodenum, jejunum, ileum, transverse colon. US features of the ileocolic region. **3.** Twelve hours fasting. **4.** General anesthesia with (halothane). **5.** Three measurements *per* anatomic region (average).	Histopathology Not determined clinically	**1.** Reports on the appearance of the ileum. **2**. Establishes comparisons with dog, equine and humans. **3.** Reports full wall normal thickness of stomach (inter-rugal folds), duodenum, jejunum, ileum, and transverse colon.	**1.** No comparison with Newell *et al.*, 1999. **2.** No assessment of stomach distension. **3.** No measurements of stomach rugal folds. **4.** Unknown influence of anesthesia with halothane in GIT wall thickness. **5.** Results with limited statistical value (low numbers, nonuniform

		6. Cross-sectional images. **7.** 10 MHz Probe.			distribution of males and females; slightly oblique positioning which may affect significantly thinner colon and significantly thicker ileum results). **6.** Unknown age.
Winter *et al.*, 2013	*n*=38 0.5 to 16 years No gender 5.2 ± 1.5 kg Alive	**1.** Muscularis, submucosal, mucosal and serosal layers thickness of the **2.** Gastric fundus, body and pyloric antrum, duodenum, jejunum, ileum, colon. **3.** Determines the ratio of muscularis (Musc:Ao) and mucosal (Muc:Ao) layer thickness to aortic diameter measured at the level of the celiac artery. **4.** Establishes the type of food ingested (wet and/or dry). **5.** Twelve hours fasting. **6.** Sedated with ketamine hydrochloride (4 mg/kg, IV) and diazepam (0.2 mg/kg IV). **7.** Three measurements per each layer (average). **8.** Transverse plan. **9.** 5-17 MHz Probes.	No histopathology Without clinical evidence of GI disease (weight loss, inappetence, vomiting, previous diagnoses of neoplasia) or evidence of other chronic disease Complete blood count and serum biochemical analysis	**1.** Reports baseline layer thickness measurements in each segment of the GI tract. **2.** Musc:Ao and Muc:Ao ratios are clinically relevant values that can be used to objectively identify thickening of the muscularis and mucosal layers. **3.** There was no correlation between age and GI layer thickness. **4.** Cats fed with a combination of wet and dry food had a small, but significantly greater, mucosal layer thickness in the ileum than cats fed with dry food only.	**1.** Some of the measurements made were at the limits of the spatial resolution of the transducer, specifically those of the serosal and submucosal layers, and the accuracy of these extremely small measurements can be questioned. **2.** Diet was not a controlled variable in the study. Including more cats on a wet food diet alone, or on different diet formulations, may reveal differences in layer thicknesses. **3.** There was no confirmation of the normal GI trat status with full-thickness intestinal biopsies.
Donato *et al.*, 2014	*n*=20 (8 intact males, 6 neutered males, 1 intact female and 5 neutered females) 4.4 ± 0.9 kg (3 to 6 kg) 1 to 7 years (mean: 3.4 ± 2 years)	1.Jejunal, duodenal, ileal (fold and between folds) and duodenal images. **2.** Measurements of full thickness wall, mucosal, submucosal, muscular and serosal thickness (consecutive measurements	Physical examination, complete blood count, routine serum biochemical analyses, urinalysis and faecal examination for intestinal parasites.	**1**. The thickness of ileum at the level of the fold was significantly higher than the other intestinal segments. **2.** The relative proportion values provided in this study can be useful as a baseline reference when evaluating feline intestinal disorders, such as inflammatory bowel disease and round cell tumours, that can have different degrees of intestinal layer involvement.	**1.** The population of cats prospectively recruited was considered healthy only on the basis of clinical and laboratory findings. No endoscopic or surgical biopsies were taken and, therefore, histological confirmation of the absolute normality of the intestinal wall was not available.

		of each of the layer). **3**. Awake and restrained manually during the examination. **4.** Twelve hours fasting. **5.** Longitudinal and transverse (transverse was used for colon measurements) **6.** 13 MHz Probe			**2.** The measurements of each individual layer of feline intestinal wall could be influenced by observer's experience, and the evaluation of inter-observer variability was not performed in this study. **3.** The accuracy of the thickness of layers of ileum can be questioned owing to the lower number of these measurements and to the difficulty to clearly distinguish the layers for the presence of folds. Therefore, these values should be validated in a wider feline population.
Hahn *et al.*, 2017	*n*=20 (4 females, 16 male) 4.3 kg (3.8–4.6) 1–8 years (average of 3) Alive	**1.** Ultrasound appearance of the proximal and distal caecum in the asymptomatic adult cat; **2**. Correlation to endoscopic and histological findings **3.** General anesthesia (induction with diazepam and propofol and then the cats were intubated with isoflurane). **4.** Twelve hours fasting. **5.** Longitudinal **6.** 18 MHz Probe	History (no clinical evidence of gastrointestinal disease: no diarrhea, hematochezia, weight loss, vomiting or dyspraxia during the last 3 months) and physical examination.	**1.** Subclinical mild caecal inflammation could be found in asymptomatic cats. **2.** Among all measured US parameters, the most accurate one in detecting this subclinical state was the thickness of the caecal folicular layer. **3.** Ultrasonography had a higher agreement with histology than with endoscopy in the evaluation of a mildly inflamed caecum. **4**. The agreement between caecal and colonic inflammation among a single evaluation technique was unsatisfactory for all three techniques. Ultrasonography, endoscopy and histopathology are complementary to evaluate the caecum fully.	**1.**The sample of cats was relatively small and there was a majority of Siamese intact male cats, which is not representative of the feline population. The low number of cats may have led to imprecise estimations of the κ values between histological, endoscopic and US results.
Martinez *et al.*, 2018	*n*=17 (6 adult males and 11 adult females) 3.6 kg (2.8–8.6 kg) Young adults Cadavers (euthanized for reasons unrelated to gastrointestinal tract disorders, such as behavioral or	**1.** Full thickness wall and measurements of each layer (mucosa, submucosa, muscularis, and serosa) for each segment of the small intestine (duodenum, jejunum and ileum); **2**. Three measurements	Not described (but none of the cats' showed signs of GIT disease, which was supported on histopathologic evaluation by a lack of abnormal cellular	**1.** There were no significant differences between ultrasonographic measurements in longitudinal and transverse planes of intestinal specimens, except for the distal ileum at the level of the fold; **2.** There was good agreement between ultrasonographic and histologic measurements of the total wall thickness and the layers of the different intestinal segments, except at the submucosa and muscularis of the duodenum.	**1.** Extensive medical history, hematology, biochemistry, urinalysis or faecal examination, were not available for the cats studied; **2**. The US transducer resolution was not confirmed experimentally; **3.** The time between

	orthopedic problems)	*per* anatomic region; **3.** Relationship between ultrasonographic measurements in the transverse and longitudinal planes. **4.** Relationship between ultrasonographic and histologic thickness. **5.** Cadavers. **6.** Twelve hours fasting. **7.** Longitudinal **8.** 13 MHz Probe	infiltrates or other.		euthanasia and fixation of intestinal samples was approximately 1 h (a study in rats reported changes in the intestinal mucosa 40 min post-mortem due to dehydration, which induced a loss of turgidity and stiffness and a thinning of the intestinal villi); **4.** The ideal fixation time in formalin was not established and standardized for the tissues obtained and it is unclear whether different fixation times could alter the histological thickness of the samples.

CBC: Complete blood count.

Table 2. Analysis of the publications regarding US evaluation of the normal GIT in dogs.

Study	Animals	Variables / measurements	Normal GI status	Conclusions	Limitations of the study
Penninck *et al.* 1989	Group 1-4 Beagle dogs; Group 2 - 4 small dogs; Group 3: 4 large dogs Group 1 - 8-12 kg; Group 2 - 4-6 kg; Group 3 - 32-54 kg Group 1 - 13, 27, and 40 weeks of age	**1.**Appearance and mural thickness transverse and longitudinal images of the stomach, proximal duodenum, small bowel and descending colon. **2.** Evaluation of degree of distension. **3.** Awake. **4.** Transverse and longitudinal. **5**. 5.0-MHz and/or a 7.5-MHz Probes	Physical examination, laboratory work (CBC, serum chemistry, fecal examination), and lack of historical information concerning GI disease.	**1**. Standardization of the GI wall thickness measurement is possible by imaging the wall during bowel relaxation. **2.** Submucosa and subserosa/ serosa are hyperechoic due to the presence of relatively more fibrous connective tissue. **3**.No significant difference was noted between the GI wall thickness of immature and mature dogs of the same breed, nor between dogs of small, medium, and large breeds.	**1.** The small number of dogs in each category is a limiting factor for any conclusive statistical analysis. The author suggested that the measurements presented the paper should be considered only as a starting point for further investigation; **2**. Colonic measurements were often compromised by large amounts of intraluminal gas.
Delaney *et al.* 2003	*n*=231 (gender was not specified) From 2.1 to 64.0 kg (median 23.0 kg).	**1**.Two measurements of jejunum and 1 of duodenum. **2**.Dogs placed into one of five groups based on body weight. **3.** Awake. **4**. Longitudinal. **5.** 7.5 to 11 MHz Probes.	Physical exam and clinical history.	**1.** Overall, the wall thickness of the duodenum was significantly greater than that of the jejunum; **2.** As weight increased, a significant increase in duodenal wall thickness was also observed, but not in the jejunum.	**1**. No description of sex and age. **2.** Duodenum was not clearly identified in all dogs.
Stander *et al.*, 2010	*n*= 23 (8 females and 15 males) 2.3 to 5 kg (3.0 ± 0.7 kg) 7 to 12 weeks of age (mean 8.8 ± 1.8 weeks)	**1**. Stomach wall measurements between rugal folds. **2.** Intestinal wall measurements: a single measurement of the proximal descending duodenum and 2 measurements of the jejunum. **3.** Descending colon 4. Width and echogenicity of the jejunal and duodenal mucosa 5. Wall layering. **4**. Awake **5.** Sagittal and transverse. **6**. 7.5–9MHz Probes.	Physical and laboratory evaluation (peripheral blood smear evaluation and fecal analysis).	**1**. Wall thickness of the gastrointestinal segments relative to each other appears to follow similar trends in puppies vs. adults. There was no significant effect of age or weight on jejunal or colonic wall thickness and on jejunal or duodenal mucosal thicknesses. **2**.There was a significant increase in duodenal and stomach wall thickness with increase in age and weight; **3**. Duodenal and jejunal mucosal layers are the thickest of the wall layers. The mean duodenal mucosal thickness constituted 71% of the total wall thickness and the mean jejunal mucosal thickness 60%.	**1**. Ultrasonography of canine pediatric patients was challenging due to poor compliance. They were difficult to restrain and reluctant to lie stationary for the 20 min ultrasonographic examination. The noncompliance led to progressive aerophagia, which hampered assessment of some structures; **2.** Stomach and colonic wall measurements were not obtained in all the puppies due to noncompliance.

				4. In the stomach the mucosa, submucosa, and muscularis were of equal thickness, but with a thinner serosa; **5.** All colonic wall layers appeared to have a comparable thickness.	
Gladwin *et al.* 2014	*n*= 85 (gender was not specified) Adults (>12months)	**1.**Images of duodenum, jejunum and descending portion of colon. **2**. Measurements were obtained from a single transverse image of the duodenum, jejunum, and colon and included total wall thickness and thickness of the mucosa, submucosa, muscularis, and serosa; **3.** Dogs were placed into one of three groups based on body weight. **4.** Awake. **5.** Transverse.	Physical exam and clinical history: no signs of gastrointestinal tract disease (vomiting, diarrhea, anorexia, or weight loss) during the 2 months preceding the abdominal.	**1.**The mucosal layer was the thickest layer of the duodenum and jejunum **2.** There was a significant difference in thickness of the mucosal layer between small and large dogs.	**1.** Histologic examination was not performed to confirm a lack of abnormalities in the gastrointestinal tract of each dog. **2.** Small sample size in each group, which may have affected the statistical power of the reference values. **3.** All measurements were made by only 1 investigator who used still images to standardize the way in which measurements were obtained. This study design did not address interobserver variation.
Roux *et al.* 2016	*n*= 12 (6 males and 6 females) verage weight of 23.4 ± 5.2 kg (4 dogs were between 10 and 20 kg, 6 between 20 and 30 kg, and 2 between 30 and 40 kg) Assumed to be young adults	**1.**Measurement of mid-segments of duodenum, jejunum and ileum. **2.** Three measurements performed independently for each intestinal layer. **3**. Dead **4.** Histopathological correlation. **5.**Transverse **6.** 13 MHz (for total wall thickness) and 15 MHz Probes (for total wall thickness and individual layers, to correlate with the histopathology)	Not described	**1**.There were significant statistical differences between histological and ultrasonographic layer thicknesses in the small intestine of adult dogs **2.** Strong to very strong positive correlation between ultrasonographic and histological layer thickness, except for the serosa.	**1**. Experimental evaluation of the ultrasound transducer resolution was not assessed. The image resolution of post processing image viewer application was approximately 0.06 mm. Therefore, for small measurements close to the axial and imaging software resolution, such as the serosa, this could have been a potential source of measurement errors. **2.** Some of the ultrasonographic and histological small intestinal measurements appear abnormally thicker in comparison to normal reported values in dogs. The values provided should however not be interpreted on their own, but only as a comparison between the two modalities used to assess intestinal layer correlation (ultrasonography and histology); **3.** Direct comparison of intestinal wall thickness measurements between *in vivo* and *ex vivo* intestinal

					segments will likely be inaccurate, as the length and thickness of a resection specimen can change after devitalization, formalin fixation, and histological section.
Banzato *et al.* 2017	*n*= 84 (gender was not specified) At 4 weeks: 2.2 ± 0.7kg (ranging from 1 to 3.6); at 8 weeks was 4.3 ± 1.8 kg (ranging from 1.5 to 8.5); at 16 weeks:9.8 ± 5.4 kg (ranging from 3.5 to 19.9) 4, 8 and 16 weeks of age	**1**.Measurement of stomach, duodenum, jejunum and colon performed at each age in every puppy. **2**. Awake. **3.** Transverse. **4.** 8–12 MHz Probes	Physical evaluation, body weight and body condition score.	**1.** Wall thickness of the gastrointestinal segments relative to each other appears to follow similar trends in puppies vs. adults. There was no significant effect of age or weight on jejunal or colonic wall thickness and on jejunal or duodenal mucosal thicknesses. **2.**There was a significant increase in duodenal and stomach wall thickness with increase in age and weight. **3.** Duodenal and jejunal mucosal layers are the thickest of the wall layers. The mean duodenal mucosal thickness constituted 71% of the total wall thickness and the mean jejunal mucosal thickness 60%. **4.** In the stomach, the mucosa, submucosa, and muscularis were of equal thickness, but with a thinner serosa; **5.** All colonic wall layers appeared to have a comparable thickness.	**1.** Ultrasonography of canine pediatric patients was challenging due to poor compliance. They were difficult to restrain and reluctant to lie stationary for the 20 min ultrasonographic examination. The noncompliance led to progressive aerophagia, which hampered assessment of some structures. **2.** Stomach and colonic wall measurements were not obtained in all the puppies due to noncompliance.

3.3. Imaging outcomes

On US, intestinal sections present a five-layered appearance with alternating hyper- and hypoechoic layers, corresponding to the mucosal surface, mucosa, submucosa, muscularis, and serosa, respectively[21]. Five echogenic layers were identified: the innermost hyperechoic layer corresponds to the surface of the mucosa; the innermost hypoechoic layer represents the mucosa; the mid hyperechoic layer is the submucosa; the outer hypoechoic layer is the muscularis propria; and the outer hyperechoic layer is the subserosa/serosa[21] (Figure 2).

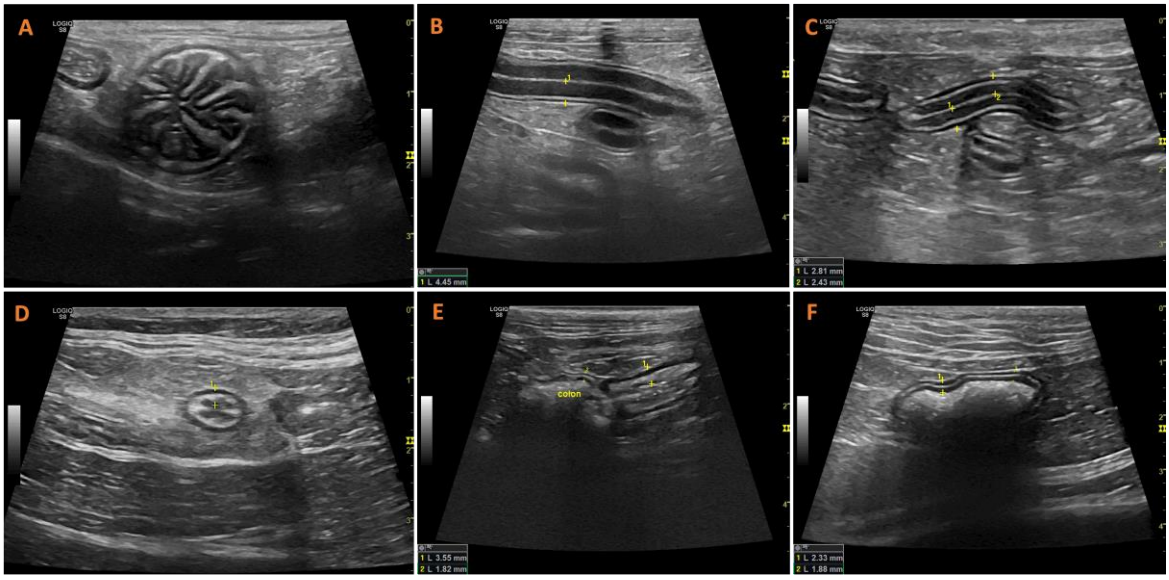

Figure 2. Ultrasonographic images of the gastrointestinal segments routinely assessed in the US evaluation of dogs and cats (GE S8, probe 10-12MHz). (A) Short axis ultrasonographic image of an empty stomach of a cat, where rugal folds are shown. (B) Longitudinal ultrasonographic images of the duodenum of a dog and (C) jejunum of a cat, evidencing the five echogenic layers: surface of the mucosa (innermost hyperechoic), mucosa (innermost hypoechoic), submucosa (mid hyperechoic), muscularis (outer hypoechoic), and subserosa/ serosa (outer hyperechoic). (D) Transverse ultrasonographic image of the distal ileum of a cat. (E) Ileocecocolic region, cat: acoustic shadowing; (F) Colon, dog: acoustic shadowing.

In the studies including dogs (*n*=6), two presented the results categorized by the weight of the animals (*n*=2/6)[19,20], one by the age (n=1/6)[17], and another one divided by weight and age (n=1/6)[21]. The stomach was evaluated in the three papers (*n*=3/6) that conducted ultrasound scans on puppies[17,22] and in one paper that study puppies and adults[21]. In this last study, there was no significant difference between the gastric wall thickness of immature and mature dogs of the same breed, nor between dogs of small, medium, and large breeds[21]. Small and large adult dogs presented gastric wall thickness of 3 mm[21]. In the studies focused only on puppies, the mean gastric thickness ranges from 2.09 mm in 4 weeks old puppies to 2.66 mm, at 16 weeks[17].

Regarding the small intestine and colon, the most recent study categorizes dogs by weight into three groups: >15 kg, 15 to 30 kg, and >30 kg[20]. The maximum mean wall thickness of duodenum (in mm) is 3.8 (< 15 kg), 4.1 (15 to 30 kg) and 4.4 (> 30 kg). The maximum mean wall thickness of jejunum (in mm) is 3.0 (< 15 kg), 3.5 (15 to 30 kg) and 3.8 (> 30 kg)[20]. However, ileum was only evaluated in one study where the mean wall thickness was 4.83 mm[18]. The maximum mean wall thickness of colon (in mm) is 1.5 (< 15 kg), 1.4 (15 to 30 kg) and 1.6 (> 30 kg) (Table 3).

In cats, the gastric wall was evaluated in the fundus, body and pylorus areas. The maximum mean values of the gastric body at the level of the rugal fold can reach up to 4.38 mm, and

2.03 mm for the inter-rugal space, with no differences according to the degree of stomach distension[10]. The mean thickness of the gastric fundus (between rugal folds) was 2.0 mm, and the pylorus mean thickness (between rugal folds) was 2.1 mm[15]. The maximum mean wall thickness described for the duodenum was 2.47 mm[13], for jejunum was 2.3 mm[15], and for ileum was 3 mm[11]. The ileocolic region presented a characteristic "wagon wheel" appearance on cross-sectional images; significantly thicker than all other portions of the GIT[15]. The maximum caecal wall thickness was 3.1 mm[14], and the maximum mean colonic wall thickness was 1.67 mm[10] (Table 4 and Figure 2).

Table 3. Range of normal gastric and intestinal segment full-wall thickness in adult dogs, according to the literature reviewed.

GI segment	**Dog range (mm)**		
	< 15kg	15-30kg	>30kg
Stomach full thickness	3.00 (trans.) and 3.30 (long.)[21]	3.00 (trans.) and 3.25 (long.)[21]	4.00 (trans. and long.)[21]
Duodenum full thickness	3.8 ± 0.5 [2.9–4.7][20]	4.1 ± 0.7 [3.0–5.5] [20]	4.4 ± 0.7 [3.1–5.7][20]
Duodenum`s layer thickness			
Mucosa	2.4 ± 0.5 [1.6–3.5][20]	2.6 ± 0.6 [1.5–3.7][20]	2.8 ± 0.5 [2.0–3.9][20]
Submucosa	0.6 ± 0.1 [0.3–0.8][20]	0.6 ± 0.2 [0.3–1.0][20]	0.6 ± 0.2 [0.3–1.2][20]
Muscularis	0.5 ± 0.1[0.2–0.8][20]	0.5 ± 0.1 [0.3–0.8][20]	0.6 ± 0.2 [0.2–0.9][20]
Serosa	0.4 ± 0.1 [0.2–0.6][20]	0.4 ± 0.1 [0.3–0.6][20]	0.4 ± 0.1 [0.2–0.7][20]
Jejunum full thickness	3.0 ± 0.5 [2.2–4.1][20]	3.5 ± 0.5 [2.4–4.8][20]	3.8 ± 0.4 [2.7–4.7][20]
Jejunum`s layer thickness			
Mucosa	1.8 ± 0.4 [1.2–2.6][20]	2.0 ± 0.4 [1.5–3.2][20]	2.2 ± 0.5 [1.1–3.2][20]
Submucosa	0.5 ± 0.1 [0.3–0.9][20]	0.6 ± 0.2 [0.3–1.0][20]	0.6 ± 0.1 [0.3–0.8][20]
Muscularis	0.5 ± 0.1 [0.2–0.7][20]	0.5 ± 0.1 [0.3–0.8][20]	0.5 ± 0.2 [0.3–0.9][20]
Serosa	0.4 ± 0.1 [0.2–0.6][20]	0.4 ± 0.1 [0.3–0.6][20]	0.4 ± 0.1 [0.3–0.6][20]
Ileum full thickness		4.83 ± 1.22 [2.51–7.18][18]	
Ileum`s layer thickness			
Mucosa		2.84 ± 0.94 [1.31–4.58][18]	
Submucosa		0.33 ± 0.1 [0.23–0.69][18]	
Muscularis		1.13 ± 0.63 [0.12–2.71][18]	

Inner muscularis		0.83 ± 0.48 [0.36–2.15][18]	
Outer muscularis		0.35 ± 0.11 [0.2–0.56][18]	
Serosa		0.19 ± 0.06 [0.07–0.33][18]	
Colon full thickness	1.5 ± 0.3 [1.0–2.0][20]	1.4 ± 0.5 [1.1–1.9][20]	1.6 ± 0.4 [1.1–2.6][20]
Colon`s layer thickness			
Mucosa	0.4 ± 0.1 [0.2–0.6][20]	0.4 ± 0.1 [0.2–0.5][20]	0.5 ± 0.1 [0.3–0.7][20]
Submucosa	0.4 ± 0.1 [0.2–0.6][20]	0.3 ± 0.1 [0.2–0.4][20]	0.4 ± 0.1 [0.2–0.5][20]
Muscularis	0.4 ± 0.1 [0.2–0.7][20]	0.3 ± 0.1 [0.2–0.5][20]	0.4 ± 0.1 [0.2–0.7][20]
Serosa	0.4 ± 0.1 [0.2–0.5][20]	0.4 ± 0.1 [0.2–0.5][20]	0.4 ± 0.1 [0.2–0.5][20]

Table 4. Range of normal gastric and intestinal segment full-wall thickness in adult cats, according to the literature reviewed.

GI segment	Cat range (mm)	
Stomach full thickness	Inter-rugal 2.03 [1.1-3.6][10]	Rugal fold: 4.38 [2.6-7][10]
Fundus	2 [1.7-2.2], 95% CI[15]	
	0.19 [0.16-0.2][12]	
Body	0.22 [0.19-0.26][12]	
Pylorus	2.1 [1.9-2.4], 95% CI[12]	
	0.21 [0.17-0.27][12]	
Stomach`s layer thickness		
Fundus		
Mucosa	0.12 [0.1-0.19][12]	
Submucosa	0.04 [0.03-0.05][12]	
Muscularis	0.06 [0.06-0.09][12]	
Serosa	0.03 [0.02-0.03][12]	
Body		
Mucosa	0.09 [0.06-0.11][12]	
Submucosa	0.04 [0.03-0.05][12]	
Muscularis	0.06 [0.05-0.08][12]	
Serosa	0.03 [0.03-0.04][12]	
Pylorus		
Mucosa	0.08 [0.06-0.10][12]	
Submucosa	0.04 [0.03-0.05][12]	
Muscularis	0.06 [0.04-0.08][12]	
Serosa	0.03 [0.02-0.03][12]	
Small intestine (no duodenum)	2.1 [1.6-3.6][10]	
Duodenum full thickness	2.2. [2-2.4] 95%	Sedated 2.71 [1.6-3.5][10*] Awake 2.4 [1.3-3.8][10*]
Duodenum`s layer thickness		
Mucosa	0.15 [0.12-0.16][12]	
Submucosa	0.03 [0.03-0.04][12]	
Muscularis	0.04 [0.03-0.05][12]	

Serosa	0.03 [0.02-0.03][12]
Jejunum full thickness	2.3 [2.1-2.5] 95% CI[15]
	2.22 [1.96-2.67][11]
Jejunum`s layer thickness	
Mucosa	0.11 [0.10-0.14][12]
Submucosa	0.03 [0.03-0.04][12]
Muscularis	0.04 [0.03-0.07][12]
Serosa	0.03 [0.02-0.03][12]
Ileum full thickness	2.8 [2.5-3.2], 95% CI[15]
	Fold 3 [2.52-3.59][11]..Between folds 2 [1.66-2.27][11]
Ileum`s layer thickness	
Mucosa	0.12 [0.09-0.15][12]
Submucosa	0.03 [0.03-0.05][12]
Muscularis	0.08 [0.06-0.1][12]
Serosa	0.03 [0.03-0.03][12]
Colon full thickness	1.5 [1.4-1.7], 95% CI[11]
	1.67 [1.1-2.5][10]
Colon`s layer thickness	
Mucosa	0.04 [0.04-0.05][12]
Submucosa	0.03 [0.02-0.03][12]
Muscularis	0.03 [0.02-0.03][12]
Serosa	0.02 [0.02-0.03][12]

CI: Confidence interval; *Sedation evidenced a significant effect on duodenal wall thickness.

3.4 Main observations

In the papers focused on cats, one of the conclusions was that sedation, as a single factor, does not significantly affect any of the parameters measured of the GIT wall thickness[10]. Also, it seems that there is no correlation between age and GIT layer thickness[12] and that there are no significant differences between ultrasonographic measurements in longitudinal and transverse planes[13]. Regarding the stomach, the thickness of the rugal folds is significantly higher than the thickness of the inter-rugal regions, meaning that separate standards of normal thickness should be recognized for these two functional areas of the stomach. Also, distension of the stomach does not significantly change the thickness of the rugal folds or inter-rugal regions[10].

In dogs, studies with puppies came to conflicting conclusions. The first study found no significant effect of age or weight on jejunal or colonic wall thickness, but observed a significant increase in duodenal and stomach wall thickness with age and weight[16]. However, another study reported an increase in the wall thickness of all the gastrointestinal tracts during development. The effect of age was more pronounced on the stomach, duodenal, and jejunal wall thicknesses, and less pronounced on the colonic wall thickness, though it was still evident. Additionally, the impact of body weight was more significant on

duodenal and jejunal wall thicknesses, while a lesser effect of body weight on stomach and colonic wall thickness was observed[17]. The third study conclude that there was significant difference between the GI wall thickness of immature and mature dogs[21].

Overall, the wall thickness of the duodenum seems to be significantly greater than that of the jejunum[19]. As weight increased, a significant increase in duodenal wall thickness was also observed, but not in the jejunum[19] nor stomach[21].

3.5. Limitations

One of the first limitations described by the authors was the low numbers of animals, resulting in limited statistical value[10,14,15,20,21]. There are also some limitations regarding study population such as unknown age[13,15,18,19] or gender[10,12,17,21]. Half of the studies used animals that were considered healthy based solely on their history, clinical and laboratory findings (with no signs of gastrointestinal disease). No endoscopic or surgical biopsies were taken; therefore, histological confirmation of the absolute normality of the intestinal wall was not available[11,12,16,17,19,20]. Out of the 6 studies involving cats, in 4 of them, the animals were either sedated or under anesthesia. Therefore, there is an unknown influence of anesthesia/sedation on GIT wall thickness[10,12,14,15]. This was not a limitation in dog studies, as the animals were awake and physically restrained. However, in the puppy studies, non-compliance resulted in aerophagy, making it difficult to observe the stomach[16] and colonic wall[16,21]. Another limitation described was the difficulty to observe some structures such as stomach wall layers[17], duodenum[19] and ileum[11].

4. Discussion

Diagnostic ultrasound is an indispensable modality in veterinary medicine, offering a non-invasive, real-time, and dynamic approach to assessing gastrointestinal health and has emerged as a cornerstone in diagnosing intestinal diseases in dogs and cats[23]. Most GIT pathologies, ranging from inflammatory conditions to neoplastic diseases, can alter the thickness and integrity of the intestinal wall layers. As a result, abdominal ultrasound has proven to be a valuable diagnostic tool for these frequently encountered diseases[23–25]. Furthermore, obtaining a histopathological diagnosis for intestinal inflammatory and neoplastic diseases can be challenging when using aspirate, endoscopic, or US-guided biopsy samples, and even with full-thickness surgical biopsies, there is a risk of missing the affected site. This emphasizes the crucial role of accurate ultrasound diagnosis in such cases[24]. While assessing the overall thickness of the gastrointestinal wall, from the inner mucosal interface to the outer serosa layer, across different GIT segments, a crucial aspect of abdominal ultrasound examinations is the detailed assessment of individual wall layers

(serosa, muscularis, submucosa, and mucosa), since this specific evaluation can provide valuable clinically relevant insights[12] (Figure 2). Keeping this in mind, it becomes evident why well-defined values and characteristics of healthy GIT are essential. They enable veterinarians to identify deviations swiftly and accurately from the norm.

Upon reviewing literature focused on cats, we find a thorough characterization of the GIT[12] and cecum[14]. The absence of weight differentiation (since there is no difference according to weight[13]) contributes to the statistical robustness of these studies, conducting to well established values that can be use as reference. In these reports, the study population consists of cats ranging in age from 0.5 to 16 years[12], encompassing a broad spectrum from very young to elderly cats. This diversity makes it a robust representation of the feline population. While gender specifications are often lacking, and even when available, gender-based data analysis is nonexistent, it's important to note that in cats, gender differences have limited relevance due to the minor variations in body structure and weight between males and females. When considering the stomach, variations in the thickness of the rugal folds compared to the inter-rugal regions highlight the importance of employing distinct standards for evaluating these areas. Nevertheless, it's worth noting that distending the stomach does not markedly alter the thickness of the rugal folds or inter-rugal regions[10]. Measurements taken throughout the intestine exhibit a relative uniformity across various studies, which underscores a level of consistency. This uniformity not only reinforces the reliability of the data but also open doors to the establishment of reference values that can be helpful in diagnosing various pathologies in cats. Moreover, it's worth highlighting the coherent approach employed in the acquisition of images. Whether captured longitudinally or transversely, there appears to be no significant differences between ultrasonographic measurements. This consistency in image acquisition strategies enhances the overall robustness of the data, facilitating a comprehensive evaluation of the feline gastrointestinal tract.

In contrast to feline studies, the characterization of the GI tract in dogs is not as well-established. For example, sedation does not seem to significantly affect the measurement of the wall along the GIT, however it did increase the full thickness of the duodenum[10]. Another difficulty is that the studies consulted for this review do not include data from older animals and do not analyze differences based on gender. This highlights the need for more diverse research to account for age-related variations and potential gender-specific differences in canine gastrointestinal health. None of the studies classify the animals by breed, but most differentiate them by weight[16,19–21]. However, there is no consensus about the categories. Delaney *et al.*[19] divided the dogs into five groups by weight while Gladwin *et al.*[20] divided into three groups. None of the papers provide a comprehensive characterization of the entire GI tract, and the evaluation of the ileum is limited to a single

study that did not differentiate animals by weight[18]. Furthermore, it's worth noting that in adult animals, the evaluation of the stomach was only conducted in one paper published in 1989[21]. In this research, owing to the limited number of animals in each category, the authors recommend that the measurements should be viewed as an initial reference point for further investigations[21]. Nevertheless, it is possible to find a description of the ultrasound appearance of the intestinal wall[21] and reference values for every section of the GI tract, including not just the total wall thickness but also the various layers of the GI tract (Tables 3 and 4).

An extensive study that assesses the complete GI tract (stomach, duodenum, jejunum, ileum, and colon) with a substantial number of animals could be highly beneficial. Such a study could help standardize not only the weight categories but also the reference values for the overall thickness of the gastrointestinal wall and its different layers. Research concerning puppies and the existence of reference values in this age group are particularly significant, given the prevalence of enteritis in this specific age range[22]. In fact, it seems that there is an increase in the wall thickness of all the gastrointestinal tracts during development[17]. However, there is no consensus between authors.

5. Conclusion

To accurately access the gastrointestinal tract of dogs and cats, we systematically reviewed the current state of the art of its normal ultrasonographic appearance and compiled reference intervals for the overall wall thickness and individual layers of all GIT segments in adult cats, dogs and puppies. Qualitative US features of the GIT are clearly established, but miscellaneous methods may influence objective imaging outcomes with respect to quantitative analysis.

In cats, the collective findings suggest that a level of coherence exists in the measurements and imaging techniques applied, ultimately providing a foundation for reference values that can be useful as a baseline reference when evaluating feline GIT disorders. However, histologically confirmed and statistically robust studies are still required in dogs and cats in different stages of development, with different body weights (dogs) or body condition scores (cats). The studies on gastrointestinal thickness in dogs use varied weight ranges, but there is no clear agreement on what is considered normal for different weight categories or genders. One critical area is the lack of larger cohort studies that account for variables such as weight categories and gender in dogs. More comprehensive studies that segment dogs based on weight and gender would provide a clearer picture of how gastrointestinal thickness varies within these groups creating a consensual range interval, offering more precise diagnostic benchmarks.

In addition, while most studies focus on overall gastric thickness in dogs, the evaluation of specific stomach structures, such as the rugal folds and the inter-rugal spaces, remains underexplored. These components are integral to gastric function, yet little research has delved into their measurement or role in normal and pathological states. A more detailed investigation of these structures could enrich our understanding of gastric health in dogs and lead to more nuanced clinical assessments.

Furthermore, while imaging techniques offer valuable measurements of gastrointestinal thickness, only most of the cat studies have incorporated histopathological confirmation of these findings[10,13–15], while in dogs only one study includes this component[18]. The combination of imaging and microscopic tissue analysis would offer a more reliable correlation between structural measurements and underlying tissue composition. Expanding studies to include histopathological validation would enhance the accuracy of gastrointestinal assessments, ensuring that imaging results align with actual tissue conditions, which is vital for diagnosing gastrointestinal diseases.

Ultrasound evaluation of the gastrointestinal tract in cats and dogs is a valuable tool in veterinary medicine, enabling a consistent and detailed assessment of functional and structural abnormalities. These evaluations should be supported by validated reference values to ensure diagnostic accuracy. By enhancing diagnostic precision, US facilitates more informed clinical decision-making, ultimately improving patient outcomes. So, as imaging technology advances, ultrasound remains an essential, non-invasive modality for gastrointestinal evaluation in small animal practice.

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3.2. Exploring Gastrointestinal Health in Diabetic Cats: Insights from Owner Surveys, Ultrasound, and Histopathological Analysis

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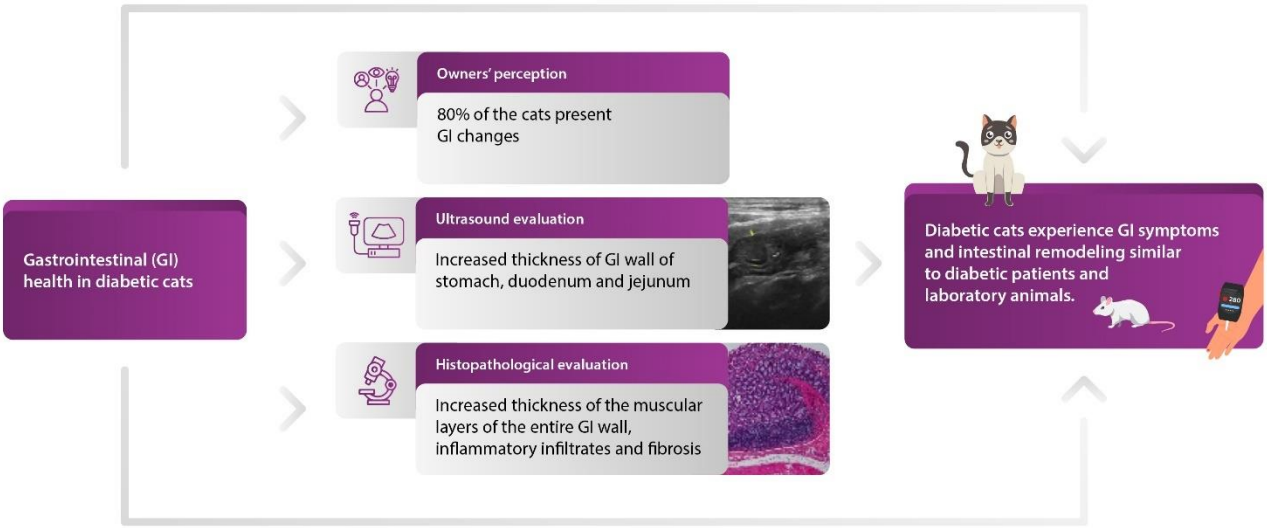
Abstract

Diabetes is a metabolic disorder characterized by chronic hyperglycemia, affecting between 0.21% and 1.24% of cats. While up to 75% of human diabetic patients experience gastrointestinal complications, there is a significant knowledge gap regarding similar changes in diabetic pets. This study sought to explore gastrointestinal alterations in diabetic cats using ultrasound and histopathological evaluations, alongside assessing owners' perceptions of digestive issues. A brief survey was conducted with the owners of diabetic cats to document diabetes signs and any observed gastrointestinal changes. Following the survey, US evaluations were performed on each cat's stomach, duodenum, jejunum, ileum, and colon. Additionally, histopathological analysis was conducted on necropsied diabetic cats. Twelve domestic spayed diabetic cats with no prior gastrointestinal disease were included, with 83% showing at least one gastrointestinal issue reported by owners. All cats exhibited increased gastric (rugal fold= 5.58±0.4 mm, Reference Value (RV)= 4.22 mm; inter-rugal= 2.82±0.08 mm, RV=2.03mm), duodenal (3.19±0.06 mm, RV= 2.20 mm), and jejunal (3.12±0.12 mm, RV=2.22mm) wall thickness. The ileum and colon showed normal thickness: 3.21±0.16 mm (RV= 3.2 mm) and 1.88±0.15 mm (RV= 1.67 mm), respectively. Histopathological evaluations revealed increased thickness of the muscular layers, inflammatory infiltrates and collagen deposits in the entire gastrointestinal tract. These findings strongly suggest that diabetic cats experience gastrointestinal signs and intestinal remodeling similar to those seen in human diabetic patients and laboratory animal models.



1. Introduction

Diabetes is a serious, chronic disorder that has become one of the fastest-growing global health emergencies of the 21st century[1]. This disease is a major contributor to mortality and morbidity worldwide, while also imposing a substantial economic burden[2,3]. Diabetes is also one of the most common metabolic diseases in domestic pets, occurring 0.21% to 1.24% of cats[4] with a higher prevalence described in Burmese cats[5]. Most spontaneous cases of diabetes occur in middle-aged to older cats[6], with obese, neutered or not, males being more commonly affected than females[7]. Similar to human diabetes, this pathology in cats is associated with high levels of mortality and morbidity[8].

While diabetes includes various forms, the two main types are type 1 diabetes (T1D) and type 2 diabetes (T2D)[9]. T1D, which accounts for 5-10% of all diabetes cases[10,11], is a chronic disease characterized by a complete absence of insulin secretion[12] due to an autoimmune inflammatory reaction targeting pancreatic β-cells[10]. On the other hand, T2D is characterized by a combination of insulin resistance in insulin-sensitive tissues and a relative deficiency of insulin production, making up 90 to 95% of all diabetes cases[10,13]. T2D emerges from a multifaceted interplay of genetic predisposition, metabolic conditions, and environmental influences[14]. Among these factors, obesity and physical inactivity coupled with the consumption of high-energy diets stands out as a significant risk factor, exerting a profound impact on T2D prevalence[15]. Although diabetic cats can present both forms of the disease, approximately 80% of them exhibit insulin-independent DM similar to T2D in humans[7]. Feline diabetes is a heterogeneous condition resulting from a combination of impaired insulin action in the liver, muscle, and adipose tissue (insulin resistance) and β-cell failure[7].

Diabetic gastrointestinal (GI) complications are highly prevalent in the human population and constitute a significant cause of morbidity, which influence the patients` health status and quality of life[16,17]. A study investigating the impact of GI complications in diabetic patients found that these significantly decrease health-related quality of life affecting not only physical functioning and general health perceptions, but also vitality, social functioning, and emotional and mental health[18]. However, awareness of these complications among physicians is often limited, with scant knowledge and treatment options available[19,20]. The entire GI tract appears to be affected by diabetes, with alterations observed from the esophagus to the rectum[20,21]. The classic GI symptoms of diabetes include post-prandial fullness with nausea, vomiting, bloating, abdominal pain, diarrhea, and/or constipation[22]. The GI tract of diabetic laboratory animals also exhibits extensive remodeling, which includes a significant increase in the thickness of the intestinal wall and the deposition of collagen[23,24]. These structural changes are indicative of underlying pathological processes

that may affect the functionality of the GI tract[24]. The increased wall thickness can lead to altered motility and nutrient absorption[25], while excessive collagen deposition may contribute to fibrosis, further compromising the integrity and performance of the intestinal lining[26].

Considering the significance of GI complications of diabetes in humans, it should be expected to find similar reports of these complications in our diabetic pets. However, there are almost no reports of GI changes in diabetic dogs and cats[27]. Diabetes has been suggested as one of the possible causes of GI dysmotility in critically ill dogs and cats[28] and in a review of cases of diabetes, seen at the Colorado State University Veterinary Teaching Hospital, about 38% of those dogs and 31% of cats also had GI disease[27].

Bearing in mind the similarities between feline diabetes and human T2D[29], as well as the lack of knowledge about GI complications in diabetic cats[27] and their potential impact on well-being, the aim of this study was to investigate whether diabetic cats exhibit GI alterations. To achieve this, we asked owners to respond to an anamnesis directed at the GI tract in order to find possible GI changes in their cats following diabetes diagnosis. Additionally, ultrasound examinations of the GI tract of diabetic cats were performed and histopathological evaluations were conducted on diabetic cats donated for *post-mortem* necropsy. The findings from this study are expected to shed light on the prevalence and nature of GI complications in diabetic cats, ultimately guiding better management and treatment strategies for these animals.

2. Materials and methods

2.1. Study population

All protocols were previously approved by local animal welfare body (ORBEA ICBAS-UP Nº381/2020).

Cats diagnosed with diabetes and receiving treatment at the Veterinary Hospital of the University of Porto (UPVet) from 2022 to 2025 were initially considered for the study (owners feedback and ultrasound evaluation). Subsequently, a rigorous selection process was implemented to ensure that the study's results would accurately reflect only the impact of diabetes on the GI tract without confounding factors. Accordingly, all animals were subject to physical examination and medical records were thoroughly reviewed. The exclusion criteria were the following: i) pre-existing GI diseases, such as inflammatory bowel disease or GI neoplasia; ii) signs of GI changes prior to the diagnosis of DM - such as vomiting, diarrhea, anorexia, or weight loss; iii) previous treatments with corticosteroids, non-steroidal anti-inflammatory drugs, or antimicrobials within 30 days before undergoing the abdominal ultrasonographic examination[30]. After careful consideration, thirteen cats were selected for

participation in the study. The sample size was calculated using the ClinCalc LLC® 2020 software to achieve 80% power and a significance level (α) of 5%, based on an expected 30% increase in diabetic cats compared to the general population.

For the histopathological analysis, five diabetic cats that died or were euthanized from causes unrelated to the GI tract and were donated to ICBAS-UP were included. Of these cats, only one had previously undergone an abdominal ultrasound. The institutions that provided these animals for necropsy were contacted to ensure that these cats had no history of GI disease and to evaluate their complete medical records. During the same period, nine non-diabetic cats that died from diseases not affecting the GI tract and had intact GI tracts were randomly selected as controls. Animals showing GI alterations, such as the presence of parasites, were excluded during necropsy. These control cats were selected to closely match the diabetic cats in age and body weight, although it has already been shown that in adult cats, age, weight, or size does not affect the thickness of the GI tract[31]. The absence of GI disease was confirmed through histopathologic evaluation, which showed no abnormal cellular infiltrates or other anomalies.

2.2. Owners' perception of digestive changes

The owners of the thirteen diabetic cats that were included in this study were given informed consent and were asked to complete a simple, yet comprehensive, anamnesis directed to the GI tract. This survey consisting of 27 questions with an estimated time of completion of 10 minutes was meticulously designed to collect detailed information about any observed digestive changes following the diagnosis of diabetes. It covered the typical signs of diabetes (polydipsia, polyuria, polyphagia and weight loss) and included specific questions about common symptoms associated with GI distress in cats, such as vomiting, diarrhea, and changes in appetite and bowel movements. A section was included at the end of the survey for owners to mention any additional GI-related observations they felt were relevant but were not specifically addressed in the previous questions.

2.3. Ultrasound evaluation of the GI tract

The selected diabetic cats underwent a comprehensive ultrasound evaluation of their entire GI tract done by an experienced veterinary radiologist. The General Electric Logiq S8 ultrasound machine was used to perform the examinations in the longitudinal and transverse planes using a 9-11 MHz linear probe. The stomach (including the rugal and inter-rugal folds), duodenum, middle jejunum, distal ileum, and distal colon walls were observed, with three separate ultrasound evaluations performed for each portion[31] for each

cat. The results were compared to standard normal reference values for GI wall thickness as documented in the literature[32–34].

2.4. Necropsy and histopathology

Both diabetic and non-diabetic cats selected as controls, which were donated for *post mortem* necropsy, underwent a thorough examination and portions of the GI tract were collected for histopathological evaluation. An experienced veterinarian pathologist performed the necropsies, paying close attention to the pancreas and GI tract. Photos of relevant lesions were taken, and a detailed necropsy report was prepared.

Samples (2 cm) of the stomach, proximal duodenum, middle jejunum, distal ileum, and distal colon were collected for histopathological analysis. These samples were routinely processed and paraffin-embedded, cut into 3 µm-thick sections, and stained with hematoxylin-eosin (HE) for histological evaluation. Each section was examined under an optical microscope (Nikon, model Eclipse E600, Nikon Instruments, Miami, FL, USA) and photographed in four different representative regions using objective lenses of 2x and 4x (magnifications of 20x and 40x). The images were used to measure the thickness of the mucosa, submucosa, circular muscle, and longitudinal muscle layers. Measurements were conducted blindly by the same person using NIS-elements software. For each sample, the layer thickness was measured at twelve different locations and averaged (three measurements *per* photo). When possible, measurements were only taken from images where the entire intestinal wall could be observed. Additionally, Masson's trichrome staining was employed to detect fibrosis in the stomach and intestinal tissues.

2.5. Statistical Analysis

The GraphPad Prism©8.1.2 software was used for statistical analysis of data. The unpaired Student's t test was used for comparison between the two groups (diabetic and control) since the variables had a Gaussian distribution. Data expressed as mean ± SEM, percentage (%) or median, as appropriate, while "*n*" refer to the number of cats in each group. The two-way ANOVA was used in the data from histopathological evaluation. In all cases, a *p* value of less than 0.05 was considered to denote a statistically significant difference.

3. Results

3.1. Study population

Of the 13 cats that participated in the ultrasound study, 4 were females and 9 were males; all were sterilized. In terms of breed, all but two were European Shorthair, with the exception being a Siamese cat and a Norwegian Forest cat. The average age was 12.5 ± 1.17 years (range: 7-19 years) and the average weight was 5.61 ± 0.65 kg (range: 2.75-9 kg). As expected, among the 13 cats, only 1 was underweight (score 3) and 4 had a normal body condition (score 5), while 3 were overweight (scores 6 and 7), and the remaining 5 were obese (scores 8 and 9), according to the WSAVA body condition scoring system for cats[35]. The duration since diabetes diagnosis ranged from 7 days to 5 years, with a median of 2 months. The average blood glucose level measured before the ultrasound was 371.56 ± 45.99 mg/dL, with a range of 170 to 600 mg/dL. The upper limit of 600 mg/dL corresponds to the maximum reading capability of the glucometer used. This cat with this value had uncontrolled diabetes and was euthanized a few days after the ultrasound.

As expected, all cats were receiving treatment to control diabetes. Caninsulin® and Lantus® were the most used insulins (5 cats each), Prozinc® (1 cat), and Degludec® (1 cat). Interestingly, only one cat was receiving a non-insulin treatment, which involved the administration of metformin. Only one cat was also receiving treatment not directed at diabetes, which was Impromune®, since this cat was positive to Feline Immunodeficiency Virus (FIV). Additionally, two other cats had health issues besides diabetes. One cat had chronic pancreatitis and was beginning to show signs of heart disease. The other cat was experiencing blindness and had degenerative lesions in the kidneys and liver. In both cases, no relationship was established between these other health issues and DM.

Regarding typical signs of diabetes, all but one owner reported the expected polydipsia and polyuria. However, only eight owners recognized polyphagia, while weight loss was observed in ten cats.

All demographic information related to the thirteen cats enrolled for this study and their typical diabetes signs are summarized in Table 1.

Table 1 – Basic animal identification data and typical diabetes signs are presented as mean ± SD (age, weight and glycemia), median (time since diabetes diagnosis) or percentage (%), as appropriate (n=13 cats). FIV – Feline Immunodeficiency Virus.

	Animal Data
Gender	9 males (69.23%) 4 females (30.77%)
Age	12.5 ± 1.17 [7-19 years]
Weight	5.61 ± 0.65 [2.75-9 kg]
Body condition	Underweight (7.69%) Normal (30.77%) Overweight (23.07%) Obese (38.46%)
Time since diabetes diagnosis	2 months [7 days to 60 months]
Glycemia	371.56 ± 45.99 mg/dL [170 - 600 mg/dL]
Diabetes treatment	Caninsulin® (38.46%) Lantus® (38.46%) Prozinc® (7.69%) Degludec® (7.69%) Metformin® (7.69%)
Comorbidities	FIV (7.69%) Chronic pancreatitis (7.69%) Blindness (7.69%) Kidney and liver degenerative disease (7.69%)
Typical diabetes signs	Polydipsia (92.31%) Polyuria (92.31%) Polyphagia (61.54%) Weight loss (76.92%)

3.2. Owners' perception of digestive changes

Out of the thirteen owners that completed the anamnesis, eleven reported at least one digestive change in their diabetic animals, representing a prevalence of digestive alterations of 84.62%. Some cats exhibited either gastric or intestinal changes, but the majority experienced both.

Regarding the upper GI tract, six owners indicated that their cats went from not vomiting to consistently vomiting either around the time of diabetes diagnosis or afterward, with a related frequency of at least two to three times *per* week. Two of these owners noted that

vomiting typically occurred within 30 minutes after a meal, and the cats maintained their appetite post-vomiting.

In terms of defecation habits, four owners reported an increase in defecation frequency, while seven reported an increase in stool volume. Only one owner reported constipation, with decreased defecation frequency. Diarrhea was described in seven animals, and tenesmus was noted in three. Six of the thirteen cats exhibited behavioral changes regarding defecation, starting to defecate outside the litter box, often on the floor. Among these six cats, three presented with diarrhea, one also exhibited an altered appetite, beginning to reject the usual solid foods, and another one started vocalizing during defecation. One owner specifically described that their cat nearly stopped using the litter box entirely for defecation.

Concerning fecal appearance, in addition to increased volume, owners reported various changes such as stronger odor (one cat), watery feces and yellowish color when defecating outside the litter box (one cat), darker color (one cat), and larger, thicker stools (one cat). In the open-ended section of the survey, one owner mentioned that their cat initially experienced constipation during the early months of diabetes, which subsequently evolved into diarrhea.

The main results related to owners' perception of digestive changes are summarized in figure 1.

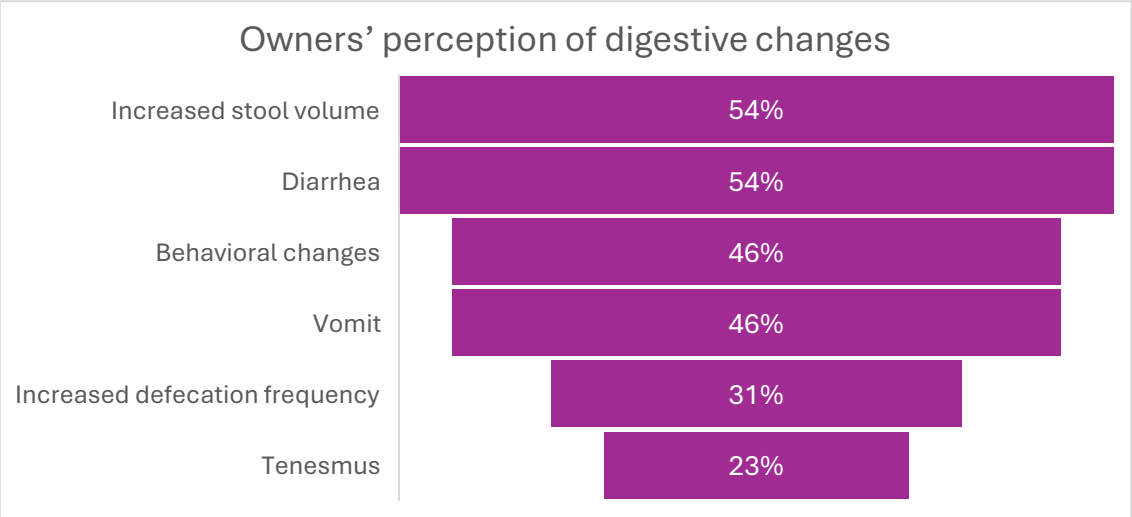


Figure 1 - The percentage (%) of digestive alterations reported by owners of diabetic cats (n=13). Behavioral changes primarily involve defecation outside the litter box.

3.3. Ultrasound evaluation of the GI tract

On average, compared to the maximum reference values (RV) documented in the literature, cats exhibited increased thickness of the gastric rugal fold (5.58 ± 0.4 mm *vs* RV:4,22 ±

0.31 mm[34]) and inter-rugal (2.82 ± 0.08 mm *vs* RV:2.03 ± 0.41 mm[34]) (figure 2). This increased in the thickness of the GI wall was also observed in the duodenum (3.19 ± 0.06 *vs* RF: 2.20 ± 0.17 mm[32]) and jejunum (3.12 ± 0.12 *vs* RF: 2.22 ± 0.18mm[32]) (figure 2). On the other hand, the ileum and colon walls displayed normal thickness in diabetic cats (ileum: 3.21 ± 0.16 mm *vs* RF: 3.00 ± 0.28mm[32]; colon: 1.88 ± 0.15 *vs* RF: 1.67 ± 0.20 mm[34]).

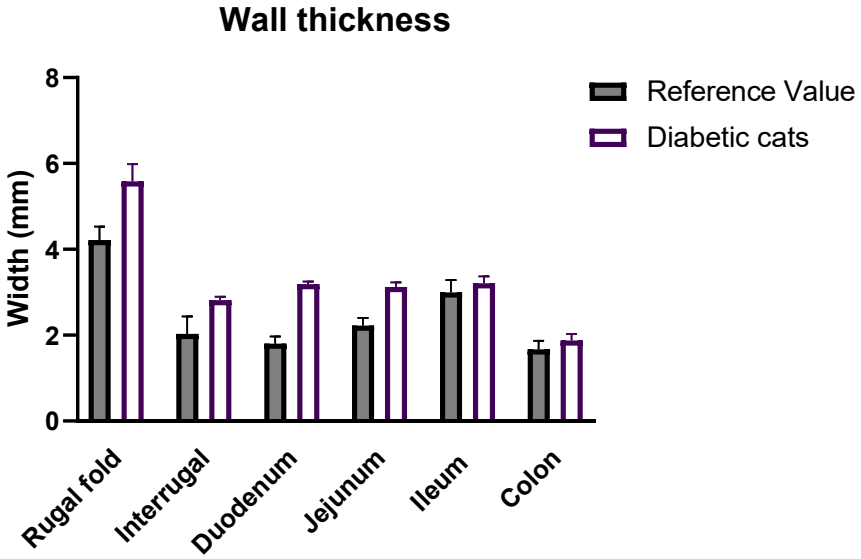


Figure 2 - Total wall thickness (mm) of the stomach (rugal fold and inter-rugal), duodenum, jejunum, ileum, and colon measured using ultrasound in diabetic cats (n=13) compared to reference values previously documented in the literature.

Regarding the stomach, ultrasound measurements of the gastric wall at the level of the rugal fold ranged from 3.99 mm to 7.97 mm. Only one cat had average values within the normal reference range, while all the others presented values above the reference range. Three diabetic cats showed an average of the three measures above 6 mm (representative image in figure 3A), which is typically considered pathological[32,33]. The inter-rugal thickness varied from 2.48 mm to 3.32 mm, and all the diabetic cats had average values from the three measurements above the reference range (3B).

In the duodenum (figure 3C), values ranged from 2.98 to 3.55 mm, with all animals presenting values above the reference range. The same was true for the jejunum (figure 3D), where values ranged from 2.63 to 3.66 mm.

The ileum was not easily visualized if filled with gas, meaning that it was not evaluated in all the diabetic cats. The veterinary radiologist was able to confidently measure the ileum in eight cats, with values ranging from 2.82 to 4.05 mm (figure 3E). Three cats presented values above the reference value.

Although the average colon measurements did not differ significantly from the normal reference values, the majority of the cats had values above 2 mm (figure 3F). The measurements for the colon ranged from 1.24 to 2.62 mm.

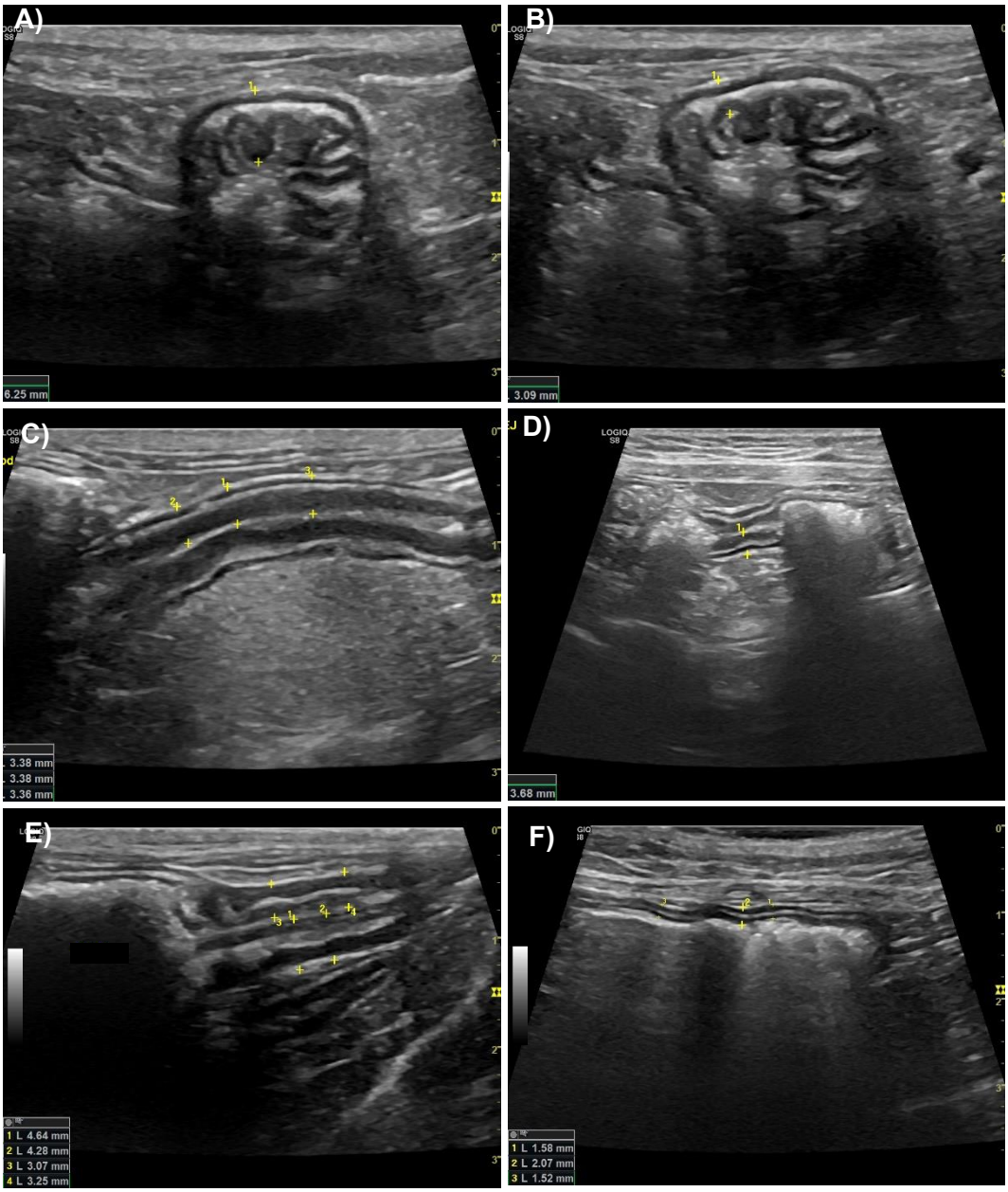


Figure 3 – Representative ultrasound images of the stomach rugal fold (A) and inter-rugal (B), duodenum (C), jejunum (D), ileum (ileo-colic transition) (E) and colon (F) of diabetic cats, longitudinal plans, using a 9-11 MHz probe. In these images all portions except colon present wall thickening compared to reference value (RV) (RV: stomach rugal fold=4.22mm; stomach inter-rugal=2.03 mm; duodenum=2.20mm; jejunum=2.22mm; ileum=3.20mm; colon=1.67mm).

In a normal GI ultrasound, five echogenic layers are identified: the innermost hyperechoic layer corresponds to the surface of the mucosa; the inner hypoechoic layer represents the mucosa; the middle hyperechoic layer is the submucosa; the outer hypoechoic layer is the muscularis propria; and the outermost hyperechoic layer is the subserosa/serosa[36]. Although there was sometimes an increase in overall wall thickness, normal GI mural stratification was preserved in all ultrasound examinations, allowing for clear identification of the previously described layers.

3.4. Necropsy and histopathological evaluation

Necropsies were performed on six diabetic cats and nine controls. Of the diabetic animals only one was previously observed and submitted to an ultrasound examination by our research team. The remaining four were donated by other veterinary clinics. The cats were all European Shorthair, ranged in age from 10 to 14 years and included four males and two females. Four of these animals were euthanized due to diabetic ketoacidosis, chronic kidney disease, and pulmonary failure, and two died spontaneously. None of these animals had a history of GI disease. Of the nine cats used as controls, six were euthanized due to various conditions: FIV (*n*=1/9), pulmonary metastasis from mammary gland tumors (*n*=1/9), high rise syndrome (*n*=1/9), and renal failure due to chronic kidney disease (*n*=3/9). The remaining three cats died spontaneously, not having a determined cause of death. During necropsy, all animals presented with an intact and healthy GI tract.

During necropsy, we found fecalomas in the colon and rectum of one diabetic cat and megaesophagus in another. Upon opening the intestinal segments, all the diabetic cats appeared to have thickened walls, as we were unable to completely open the segments; the walls maintained their structure (figure 4A green arrow). Additionally, several areas of hyperemia were observed in the mucosa characterized by a reddened appearance (figure 4A purple arrows). These hyperemic regions were distributed throughout various segments of the intestinal tract, indicating localized inflammation.

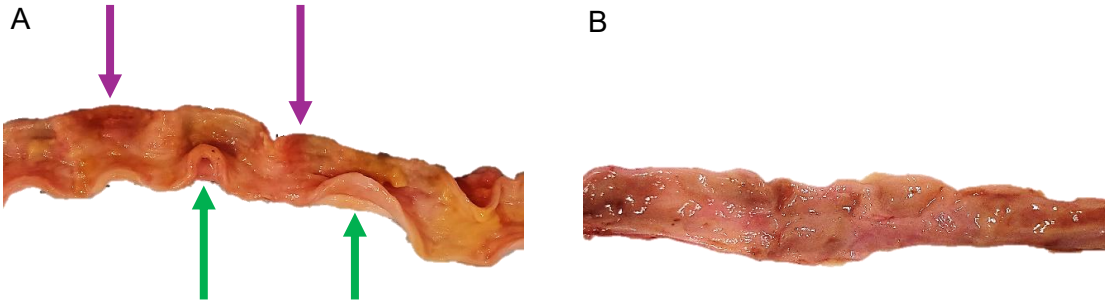


Figure 5 – Photos taken during intestinal necropsy of: A) Diabetic cat - jejunal wall exhibited significant thickening, curling upon opening of the intestine rather than falling as expected (green arrow), accompanied by areas of hyperemia (purple arrows); B) Control cat - normal jejunal wall.

Microscopic evaluation revealed that the GI wall was thickened in the stomach (3016.97 ± 486.20 µm *vs* 2198.38 ±75.58 µm, *p*=0.0335), duodenum (2108.74 ± 175.27 µm *vs* 1593.73 ± 68.28 µm, *p*= 0.0279), and jejunum (1781.49 ± 81.08 µm *vs* 1239.89 ± 64.60 µm, *p*= 0.0007) of diabetic cats compared to controls (figure 6A). This difference was not observed in the ileum (2409.85 ± 141.72 µm *vs* 2111.52 ± 93.69 µm, *p*= 0.1561) and colon (1479.79 ± 163.23 µm *vs* 1390.39 ± 111.40 µm, *p*= 0.6641) of diabetic cats compared to controls (figure 6A).

Notably, the muscular layers were consistently increased across all studied sections in the diabetic cats, compared to controls (stomach – longitudinal muscle: 273.18 ± 34.02 µm *vs* 158.67 ± 11.34 µm, circular muscle: 1083.77 ± 237.35 µm *vs* 483.25 ± 58.72 µm; duodenum - longitudinal muscle: 186.36 ± 17.99 µm *vs* 126.54 ± 9.96 µm, circular muscle: 750.00 ± 90.48 µm *vs* 315.77 ± 33.84 µm; jejunum - longitudinal muscle: 184.98 ± 16.41 µm *vs* 92.93 ± 8.47 µm, circular muscle: 546.34 ± 52.49 µm *vs* 250.11 ± 12.61 µm; ileum - longitudinal muscle: 305.00 ± 26.69 µm *vs* 186.42 ± 19.39 µm, circular muscle: 800.46 ± 29.76 µm *vs* 492.29 ± 28.74 µm; colon - longitudinal muscle: 323.53 ± 45.41 µm *vs* 198.79 ± 23.84 µm, circular muscle: 237.38 ± 58.85 µm *vs* 170.98 ± 20.68 µm, respectively, *p*<0.05 for all). However, the mucosal layer showed a significant increase only in the jejunum of the diabetic cats (873.38 ± 25.96 µm) compared to control cats (737.23 ± 39.98 µm) (*p*= 0.0178).

Representative microscopic photographs of all the intestinal segments of control and diabetic cats stained with hematoxylin and eosin are shown in figure 7. In addition to the quantitative analyses, a qualitative assessment was performed by an experienced pathologist. Diabetic cats exhibited inflammatory infiltrates throughout all sections of the GI tract, which were absent in control animals (figure 7).

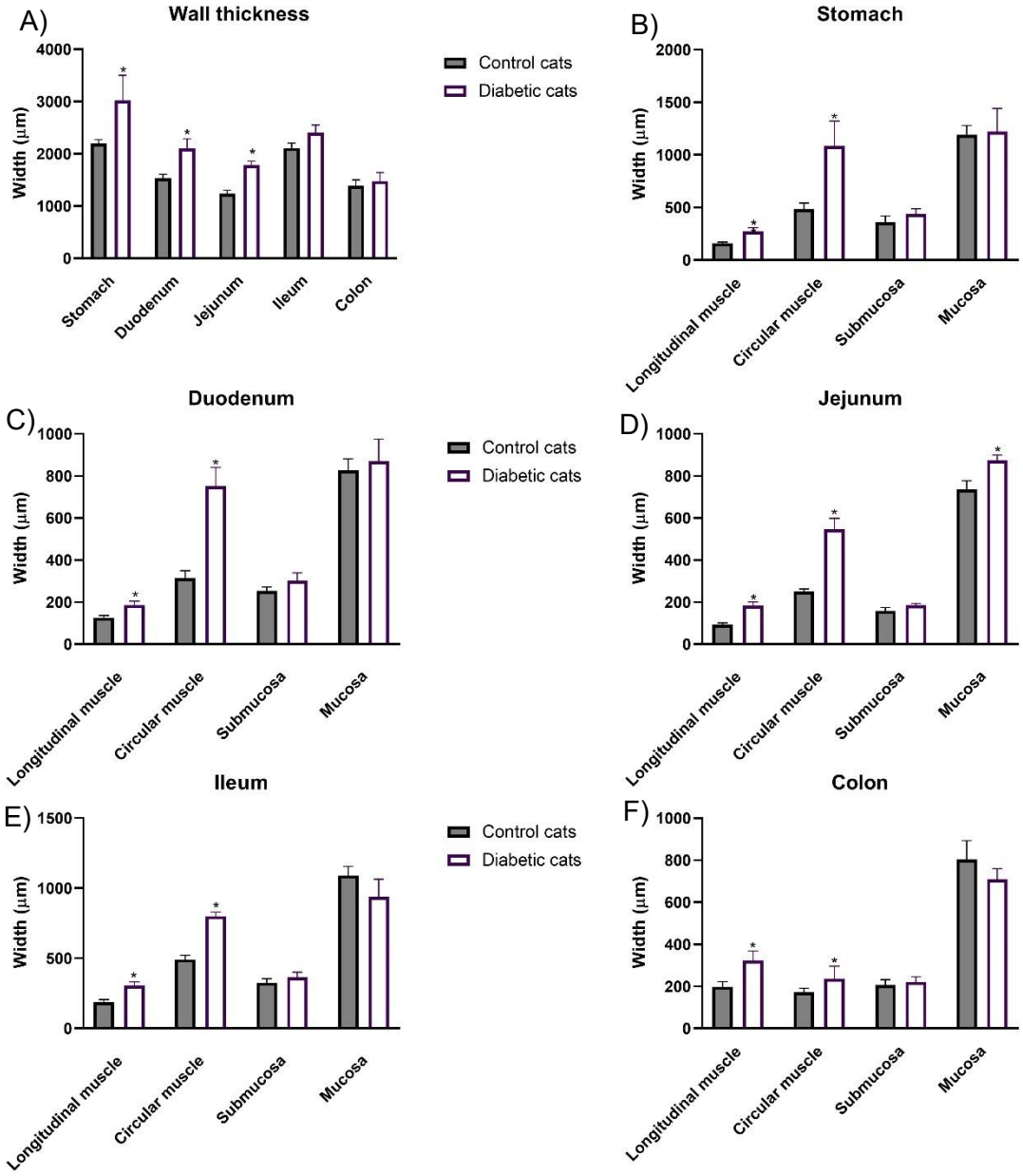


Figure 6 - Morphometric evaluation of intestinal segments (stomach, duodenum, jejunum, ileum and colon) of control cats (n=9) and diabetic cats (n=6). Total wall thickness (µm) of each intestinal segment (A); thickness (µm) of the intestinal layers (longitudinal muscle, circular muscle, submucosa and mucosa) of stomach (B), duodenum (C), jejunum (D), ileum (E) and colon (F). Values are mean ± SEM and a 2-way ANOVA followed by an unpaired t test with Welch's correction was used to compare the two experimental groups (control and diabetic cats). * Statistical difference $p < 0.05$ vs. correspondent control.

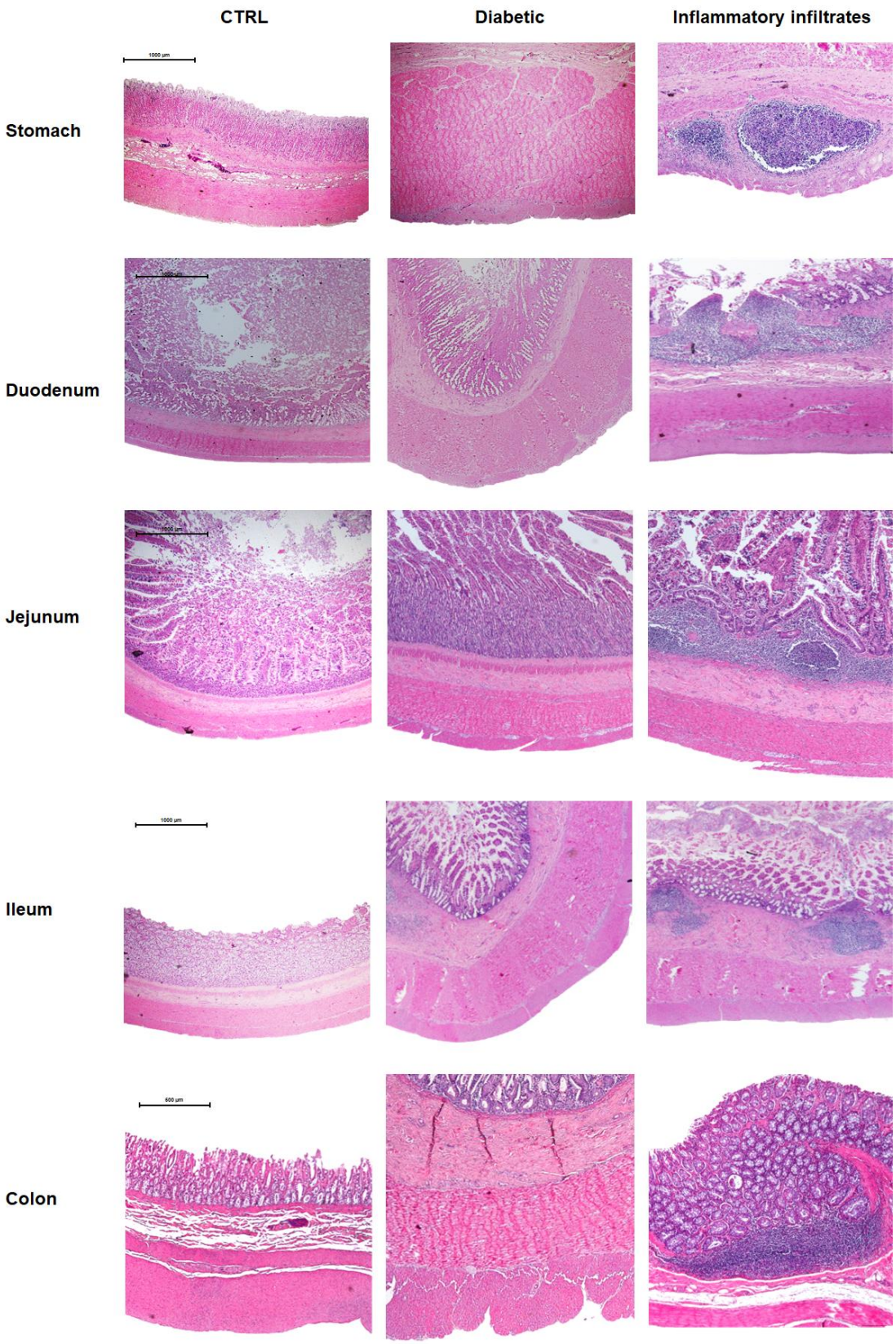


Figure 7 - Representative microscopic images of all intestinal segments from control and diabetic cats, stained with hematoxylin and eosin, as well as inflammatory infiltrates (IF) observed throughout the gastrointestinal tract of diabetic cats. All images were captured at 20x magnification, except for the colon, which was captured at 40x.

Furthermore, Masson's trichrome staining revealed abnormal collagen deposits across all intestinal segments studied, with a particularly pronounced accumulation in the muscular layers of diabetic cats. This staining technique differentiates collagen from other tissue components by coloring it blue, while muscle fibers appear red37. The blue patches observed within the muscular layers of diabetic cats indicate collagen deposition, suggesting fibrosis and structural remodeling within the intestinal wall. Representative images are shown in figure 8.

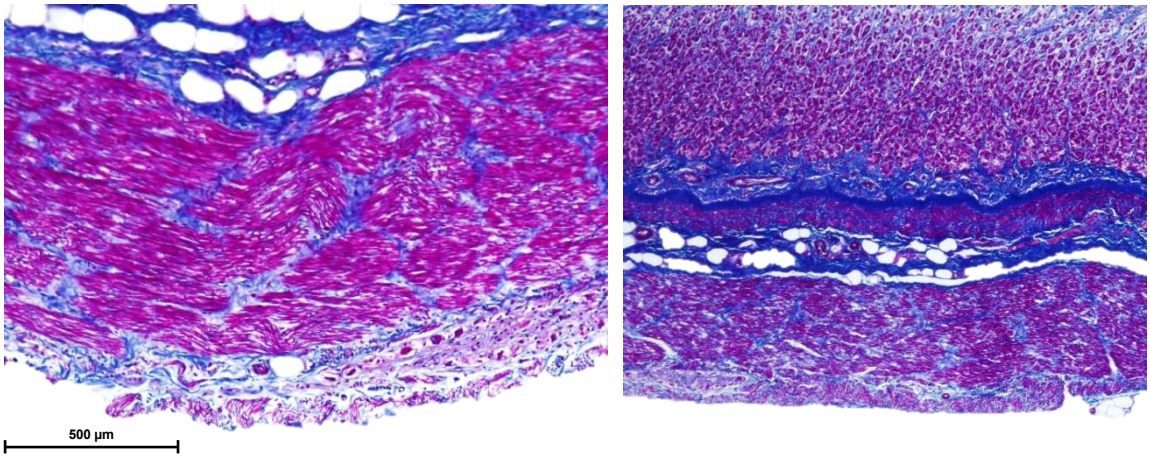


Figure 8 - Representative microscopic images of jejunum (left) and stomach (right) diabetic cats, stained with Masson's trichrome. The blue patches within the muscular layers of diabetic cats indicate collagen deposition. Images captured with a 40x magnification.

4. Discussion

This study marks the first exploration into the GI health of diabetic cats, unveiling intriguing parallels with human diabetes. The findings suggest that diabetic cats may experience similar signs to those observed in humans, with 83% of cat owners reporting noticeable digestive changes. Ultrasound evaluations revealed significant thickening of the GI wall, while histopathological analysis uncovered widespread fibrosis and inflammatory infiltrates throughout the GI tract.

All demographic data of the cats enrolled in this study align with expectations. Most cases of spontaneous diabetes occur in middle-aged to older cats (10-14 years)[6], and the average age of the cats that underwent ultrasound falls within this range, as well as all the necropsied diabetic cats. The fact that most of the animals were neutered obese males is also consistent with existing literature[7]. Male cats are significantly more predisposed to diabetes compared to females, due to gender differences in weight gain and insulin sensitivity[38].

Additionally, male cats are more prone to weight gain, are more negatively affected by it, and have higher basal insulin levels with lower insulin sensitivity[39]. Similar to findings in human medicine, obesity, together with physical inactivity, are believed to be the main contributors to the insulin resistance associated with diabetes in cats[38,40]. Therefore, it is understandable that more than 60% of the cats in this study were overweight or obese. Additionally, contrary to what is described in dogs[41], neutering is a risk factor because the cats become more prone to becoming overweight, as gonadectomy reduces energy requirements and increases voluntary food consumption[40].

Diabetic chronic hyperglycemia leads to elevated glucose levels in the glomerular filtrate, and the presence of unabsorbed glucose acts as an osmotic solute in urine, causing osmotic diuresis, polyuria, and thirst, resulting in increased water intake[42,43]. It is therefore unsurprising that all owners but one reported observing polydipsia and polyuria in their pets. In individuals with diabetes, despite high blood glucose levels, there is a lack of glucose uptake by the cells leading to reduced body mass and weight loss[43] and polyphagia emerges as a compensatory response[44]. This explains simplistically why most cats also present polyphagia and weight loss.

The glycemia values of cats included in this study indicate poor glycemic control. Most of the cases involved animals admitted to UPVet due to high glycemic episodes. Diabetic companion animals' owners usually report difficulties in managing and administering treatment to their diabetic animals, and that impact their daily routines and quality of life, representing not only a temporal but also a financial burden[45]. This challenge in maintaining proper treatment likely explains why only one cat in the study had been diagnosed with diabetes for five years and another for 2 years, while the remaining cats had been diagnosed with diabetes for only a few months or days. This aligns with the literature, which states that 1 in 10 cats is euthanized at the owner's request at the time of diabetes diagnosis[46], being the mortality rate in diabetic cats within the first 3-4 weeks of 11-17%[47]. A more recent study found that the median survival time for diabetic cats was 516 days, with a range of 1 to 3468 days[48]. When considering euthanasia, owners reported that concurrent disease, costs, and age were the most important factors[45]. Hence, it makes sense to look at the possible impact of diabetes in the GI tract of diabetic cats. Interestingly, GI complications of diabetes appear to affect up to 75% of diabetic human patients[16,17], and this study suggests they may affect more than 80% of diabetic cats. Some of the most common GI symptoms of diabetes in human population are vomiting (mostly due to gastroparesis), constipation, diarrhea, and fecal incontinence[20,49]. So, it's not surprising that the most common digestive changes described by the owners of diabetic cats are vomiting and diarrhea.

Gastroparesis in diabetic human patients is extensively studied but remains poorly understood[20]. Poor glycemic control seems to be enough to cause disrupts in gastric coordination and emptying,[20] and the presence of neuronal damage[50] and remodeling of the gastric wall[51] are also identified risk factors. Indeed, a decreased number and phenotypic changes of myenteric neurons[50], a decrease expression of nitrergic neurons and reduced number of Interstitial Cells of Cajal[52] have been linked to gastroparesis and vomiting[53] in laboratory animals and humans. In companion animals, a single study evidenced a notable reduction in the density of nitrergic neurons in both the antrum and ileum of diabetic dogs compared to the control group[54].

One common observation in diabetic cats was also an increase in fecal excretion. This aligns with our own research, which also noted increased fecal excretion in STZ-induced diabetic animals. These findings may be attributed to polyphagia and intestinal distension[23]. Furthermore, diarrhea in diabetic patients is multifactorial and may involve the accumulation of advanced glycation end-products, neuronal damage, and remodeling of the intestinal wall, especially fibrosis of the muscular layers[24]. It is typically intermittent, watery, painless, nocturnal, and may be associated with fecal incontinence in at least a third of the patients[55,56]. The fact that almost half of the owners (6/13) reported that the cats started to defecate outside the litter box may indicate that these animals also suffer from fecal incontinence. In human patients, episodes of incontinence are considered a troublesome symptom and may be attributed to the anal sphincter dysfunction and neuronal damage, potentially exacerbated by acute hyperglycemic episodes[57], that inhibit the sphincters and reduce rectal compliance[20,58]. Feline fecal incontinence usually suggests neurologic-related anal sphincter incontinence[59]. In diabetic patients this symptom indicates poor glycemic control[57] raising the question of whether the suboptimal glycemic control observed in the cats in this study may also contribute to the alteration in defecation habits. Considering the burden of caring for diabetic cats on their owners, having the cats defecate outside the litter box can represent a significant additional challenge[60]. Discovering feces at home can be a significant source of frustration for cat owners as it is considered unpleasant and unhygienic to live with a pet with this condition[59,60]. This problem demands both time and financial investment in cleaning and possibly repairing surfaces and neglecting to address this issue can strain the bond between human and animal[60]. In fact, house-soiling is a major cause for cats being abandoned or euthanized[60,61]. Given that we observed this behavior in almost half of the cats that underwent ultrasound, the authors of this study believe it would be useful to distribute a general questionnaire to the owners of diabetic cats. This approach is essential to determine if this is, indeed, a common issue among diabetic cats.

While owners report various digestive changes in diabetic cats, it prompts the question: are there corresponding morphological changes in the GI tract of these animals?

Ultrasonography has emerged as a cornerstone in diagnosing intestinal changes in cats[62], since most of GI pathologies can alter the thickness and/or integrity of the intestinal wall layers[62–64]. In this study, we found that while the integrity of the intestinal layers is maintained, there is a thickening in the jejunum, duodenum, and stomach, with some animals exhibiting a gastric wall thicker than 6 mm. The histopathological results supported the ultrasound findings in diabetic cats, as increased thickness of the GI wall was consistently observed in the stomach, duodenum, and jejunum. However, morphometric analyses additionally revealed that the muscle layers in all studied sections were increased in diabetic cats compared to controls, primarily due to collagen deposits revealing the existence of fibrosis.

Previous studies have established that increased thickness of stomach muscle layers, due to collagen deposition, is common in both diabetic patients and experimental diabetic models (mostly rats)[65,66]. This increased thickness is responsible for greater stiffness, absorption problems, and abnormal motility of the gastric wall, potentially resulting in either faster or delayed gastric emptying, contributing to the gastric symptoms in diabetic patients[67], and possibly explaining the increased frequency of vomit in diabetic cats related by almost half of diabetic cat owners. Additionally, food retention in the stomach combined with posterior accelerated gastric emptying contributes to poor post-prandial glycemic control, leading to irregular hyper and hypoglycemic episodes[68], which can also be related to the poor glycemic control observed in the diabetic cats in this study.

The intestinal thickening found in diabetic cats aligns with what has been extensively described by other researchers, including our own research group[23,24,69,70]. The increase in the thickness of mucosa seems to be related to increased food intake[50], increased expression in diabetic animals of glucagon-like peptide-2 that as a trophic action on the intestinal epithelium[71] and suppression of apoptosis[72]. The increased thickness in the muscle layers appears to be directly related to the accumulation of Advanced Glycation End Products[70] and collagen type I[73]. The collagen fibers accumulate mostly around and between smooth muscle cells, causing stiffening of the diabetic gut and decreased resting compliance. In addition to extracellular matrix remodeling, authors also found smooth muscle cell hypertrophy[73]. This remodeling is significant as it can influence absorption, and cause small intestinal bacterial overgrowth and motility disorders, contributing to symptoms such as constipation, diarrhea, and fecal incontinence[25,74], symptoms similar to those observed in the diabetic cats included in this study. The fact that the GI wall thickening in diabetic cats is gradually less preeminent in the distal direction is also compatible to what was described by our own group[23] and Fregonesi *et al.*[50].They showed that there is a differential effect of diabetes in the GI tract, with the distal segments being affected last[50].

In addition to intestinal remodeling and fibrosis, the histopathological results also revealed the presence of inflammatory infiltrates throughout the entire GI tract, which were not consistent with inflammatory bowel disease. These findings were not surprising, as inflammatory infiltrates have previously been observed in the stomach[75], intestine[76], and colon[77] of both diabetic patients and laboratory animals. Diabetes, particularly T2D, is often associated with chronic low-grade inflammation with an increased in circulating inflammatory cytokines. This systemic inflammation is linked to insulin resistance and can affect various organs, including the GI tract by disrupting normal cellular functions and promoting inflammatory responses[78]. Additionally, diabetes can cause changes in the gut microbiota, leading to dysbiosis and the promotion of inflammation in the gut[77]. It is also associated with increased intestinal permeability ("leaky gut"), which allows endotoxins and inflammatory mediators to enter the bloodstream[79]. These inflammatory infiltrates are important as they can be associated with other GI alterations such as fibrosis and can impact gut function and further contribute to GI symptoms of diabetes[80].

This study presents some limitations that should be considered when interpreting the findings. One of the main constraints relates to the use of reference values from the literature for the interpretation of ultrasound data in the absence of an internal control group. While these values provide a useful benchmark, they cannot be directly correlated with the histopathological findings obtained. Additionally, the relatively small sample size, though acceptable for an exploratory study, may limit the statistical power and generalizability of the results. A larger sample size would be beneficial in future studies to enhance the robustness of the findings. Owner-reported clinical signs, gathered through semi-structured questionnaires, may also be subject to variability and interpretation bias. All diabetic cats in this study were reportedly fed commercial dry diabetic food, which likely helped reduce dietary variability. While these diets share similar nutritional goals, minor differences between brands and owner-reported data may still introduce some variability. The specific impact of such diets on gastrointestinal morphology is not well established and should be further explored in future studies with controlled feeding protocols. Lastly, the study focused primarily on structural and histological assessments, without integrating functional analyses such as gastrointestinal transit time or motility tests, blood biochemical markers, endotoxin levels, and alterations in gut microbiota, which could provide a more comprehensive view of the underlying pathophysiology.

This pioneering study is the first to investigate the GI health of diabetic cats, revealing significant findings that align with patterns seen in human diabetic patients. Remarkably, more than 80% of the diabetic cats in our study displayed at least one GI issue, with increased vomiting frequency, diarrhea and defecation outside the litter box being common problems. Both ultrasound and histopathological evaluations uncovered notable thickening

of the GI wall in the stomach, duodenum, and jejunum. Additionally, we observed increased thickness of the muscular layers throughout the entire GI tract, accompanied by inflammatory infiltrates and fibrosis. These findings suggest that diabetic cats experience GI signs and intestinal remodeling like those observed in human patients and experimental models of diabetes.

This research underscores the significant impact of diabetes on feline digestive health, opening new avenues for understanding and treating this condition in pets. However, further research is essential to fully grasp how these GI changes affect the quality of life for both diabetic cats and their owners. It also highlights the importance for veterinarians to consider these potential alterations when developing treatment plans for diabetic cats.

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3.3. Gastrointestinal changes in diabetic dogs: preliminary results

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Abstract

Diabetes is a metabolic disorder characterized by chronic hyperglycemia, affecting approximately 0.34% to 1.2% of dogs, with a rising prevalence. While up to 75% of human diabetic patients experience gastrointestinal complications, similar alterations in diabetic dogs remain poorly understood. This study aimed to investigate gastrointestinal changes in diabetic dogs through ultrasound and histopathological assessments, as well as owners' perceptions of digestive symptoms. A brief survey was conducted with owners of diabetic dogs to document symptoms and any observed gastrointestinal issues. Ultrasound evaluations were then performed on each dog's stomach, duodenum, jejunum, ileum, and colon. Additionally, histopathological analysis was carried out following a diabetic dog necropsy. The study included ten diabetic dogs without prior gastrointestinal disease, with 50% of owners reporting at least one gastrointestinal symptom, most commonly vomiting and diarrhea. Ultrasound findings revealed increased wall thickness in the stomach, duodenum, and jejunum, while ileum and colon measurements remained within the expected range. Histopathological examination identified inflammatory infiltrates and collagen deposits throughout the gastrointestinal tract. Although limited by the small sample size, these preliminary findings strongly suggest that diabetic dogs experience gastrointestinal symptoms and intestinal remodeling similar to those observed in human diabetic patients and laboratory animal models. Further studies with larger cohorts are needed to confirm and expand upon these results.

1. Introduction

Diabetes is highly prevalent chronic disorder with a high impact in mortality and morbidity rates worldwide[1]. Diabetes is also one of the most common metabolic diseases in dogs and the leading disorder of the endocrine pancreas[2], affecting approximately 0.34% to 1.2% of dogs[3,4]. Since 2006 the incidence of diabetes in dogs has increased by and estimated 79.7%[5]. At least one of 100 dogs reaching 12 years of age develops diabetes[6], with an even higher occurrence in genetically predisposed breeds such Samoyeds, Miniature Schnauzers, Miniature Poodles, Pugs, Toy Poodles, amongst others[5,7]. Most cases of spontaneous diabetes occur in middle-aged dogs (7-12 years)[2] and unlike in cats, intact female dogs are affected twice as often as males[8]. Canine diabetes is also associated with high mortality and morbidity rates[9], as well as a significant financial impact[5].

While human diabetes includes various forms, the two main types are type 1 diabetes (T1D) and type 2 diabetes (T2D)[10]. T1D is chronic autoimmune disease and comprises 5-10% of all cases of diabetes[11,12]. Under normal conditions, pancreatic β-cells release insulin in response to elevated blood glucose levels. However, in T1D, there is a complete absence of insulin secretion[13] due to cellular-mediated autoimmune inflammatory reaction targeting pancreatic β-cells[11]. Canine diabetes is characterized by persistent hyperglycemia and insulin deficiency due to extensive β-cell loss[5], thus resembling human T1D. The precise cause of pancreatic β cell dysfunction and destruction in dogs remains unclear[14], but evidence suggests that autoantibodies[15] previously described for human patients are also present in some diabetic dogs.

Gastrointestinal (GI) complications in diabetes are highly prevalent in the human population and constitute a significant cause of morbidity, which influence their health status and quality of life[16,17]. The entire GI tract appears to be affected by diabetes, with alterations observed from the esophagus to the rectum[18,19]. The classic GI symptoms of diabetes include post-prandial fullness with nausea, vomiting, bloating, abdominal pain, diarrhea, and/or constipation[20]. The GI tract of diabetic laboratory animals also exhibits extensive remodeling, which includes a significant increase in the thickness of the intestinal wall and the deposition of collagen[21,22]. These structural changes are indicative of underlying pathological processes that may affect the functionality of the GI tract[22]. The increased wall thickness can lead to altered motility and nutrient absorption[23], while excessive collagen deposition may contribute to fibrosis, further compromising intestinal integrity and performance[24].

Considering the significance of diabetic GI complications in humans, it should be expected to find similar reports of these complications in diabetic pets. However, there are almost no reports of GI changes in diabetic dogs or cats[25]. Only one paper from 2016 focused on possible GI changes in diabetic dogs. This study concluded that diabetes in dogs leads to

significant alterations in the myenteric neuronal composition, particularly affecting the nitrergic neuronal subpopulation[26]. Since this study, no publications have specifically addressed the GI tract of diabetic pets.

Given the similarities between canine diabetes and human T1D[27], as well as the limited knowledge about GI complications in diabetic dogs[25] and their potential impact on well-being, the aim of this study was to investigate whether diabetic dogs exhibit GI alterations. To achieve this, we asked owners to complete an anamnesis focused on the GI tract to identify potential digestive changes in their dogs following a diabetes diagnosis. Additionally, ultrasound examinations of the GI tract was performed on diabetic dogs, and a single histopathological evaluation was conducted on a diabetic dog donated for *post mortem* necropsy. This study aims to clarify the prevalence and characteristics of GI complications in diabetic dogs, providing insights to improve their management and treatment.

2. Materials and methods

2.1. Study population

All protocols were approved by the local animal welfare body (ORBEA ICBAS-UP Nº381/2020). For the anamnesis and ultrasound evaluation, the study included dogs diagnosed with diabetes who were receiving treatment at the Veterinary Hospital of the Institute of Medicine and Biomedical Sciences, University of Porto (UP-VET) between 2022 and 2024. A thorough review of medical records and physical examinations was conducted to exclude any dogs with pre-existing GI conditions, such as inflammatory bowel disease or GI neoplasia. Additionally, dogs showing any signs of GI issues prior to the diagnosis of diabetes, such as vomiting or diarrhea, were also excluded. Dogs that had received corticosteroids, non-steroidal anti-inflammatory drugs, or antimicrobials within 30 days before the abdominal ultrasound examination were also excluded[28]. This careful selection process was designed to ensure that the study's results accurately reflect the impact of diabetes on the GI tract without interference from other factors. After this rigorous selection, ten dogs met the inclusion criteria and were chosen for the study.

For the histopathological analysis, only diabetic dogs that died from causes unrelated to the GI tract and were subsequently donated to ICBAS-UP were included. To date, only one diabetic dog was submitted to necropsy and since it came from UP-VET we had access to complete medical history.

2.2. Owners' perception of digestive changes

Informed consent was obtained from the owners of the ten diabetic dogs that met the inclusion criteria. They were asked to complete a comprehensive anamnesis focused on the GI tract. The survey, consisting of 27 questions, required approximately 10 minutes to complete, and aimed to gather detailed information about any digestive changes observed following the diagnosis of diabetes. It addressed the typical signs of diabetes - polydipsia, polyuria, polyphagia, and weight loss - along with some specific questions regarding common gastrointestinal signs in dogs, such as vomiting, diarrhea, and changes in appetite and bowel movements. Additionally, a section was included at the end of the survey for owners to provide any further GI-related observations that were not specifically covered in the previous questions.

2.3. Ultrasound evaluation of the GI tract

The selected diabetic dogs underwent a comprehensive ultrasound evaluation of their entire GI tract, conducted by an experienced veterinary radiologist. The examinations were performed using a General Electric Logiq S8 ultrasound machine with a L11 probe, capturing images in both longitudinal and transverse planes. The evaluation focused on the stomach, duodenum, middle jejunum, distal ileum, and distal colon walls, with each segment being assessed through three separate ultrasound evaluations *per* dog. The results were then compared to standard normal reference values for GI wall thickness as documented in the literature.

2.4. Necropsy and histopathology

To date, only one diabetic dog has been donated for *post mortem* necropsy. The necropsy was conducted by an experienced pathologist, with particular attention given to the GI tract. Relevant lesions were photographed, and a detailed necropsy report was prepared.

Samples (2 cm) from the stomach, proximal duodenum, middle jejunum, distal ileum, and distal colon were collected for histopathological analysis. These samples were routinely processed, paraffin-embedded, sectioned at 3 µm thickness, and stained with hematoxylin-eosin (HE) for histological evaluation. Each section was examined under an optical microscope (Nikon, model Eclipse E600, Nikon Instruments, Miami, FL, USA) and photographed in four representative regions using objective lenses of 2x and 4x (20x and 40x magnifications). The images were used to measure the thickness of the mucosa, submucosa, circular muscle, and longitudinal muscle layers. Measurements were conducted using NIS-elements software, with layer thickness assessed at twelve different

locations *per* sample and averaged (three measurements *per* photo). When feasible, measurements were taken from images where the entire intestinal wall was visible. Additionally, Masson's trichrome staining was employed to detect fibrosis in the stomach and intestinal tissues.

3. Results

3.1. Study population

Of the 10 dogs included in the ultrasound study, 4 were females (three spayed) and 6 were males (four neutered). The breeds represented were three Labrador Retriever, two Yorkshire Terriers, one Epagneul Breton, one Podengo, one Cavalier King Charles Spaniel, and two mixed-breed dogs. The average age was 10.8 ± 0.65 years (range: 8-11 years) and the average weight was 16.93 ± 3.85 kg (range: 4-41 kg). Most of the dogs had a normal body condition, except for three dogs who were overweight. The duration since the diabetes diagnosis ranged from 4 days to 12 months, with a median of 3 months. The average blood glucose level measured before the ultrasound was 295.45 ± 29.67mg/dL. As expected, all the dogs were receiving Caninsulin® as treatment for diabetes. Only one diabetic dog had other health issues, which was a history of splenic tumor. Regarding the typical signs of diabetes, all but one owner reported the expected polydipsia, polyuria, and weight loss. However, only five owners recognized that their dog had polyphagia.
All the information related to demographic data and typical diabetes signs of the 10 dogs enrolled for this study are summarized in Table 1.

Table 1 – Dog's identification data and typical diabetes signs are presented as mean ± SD (age weight and glycemia), median (time since diabetes diagnosis) or percentage (%), as appropriate (n=10 dogs).

	Animal Data
Gender	6 males (60%) 4 females (40%)
Age	10.8 ± 0.65 [8-11 years]
Weight	16.93 ± 3.85 [4-41 kg]
Breed	Labrador Retriever: 30% Yorkshire Terriers: 20% Epagneul Breton: 10% Podengo: 10% Cavalier King Charles Spaniel: 10% Mixed breed: 20%

Body condition	Normal: 70% Overweight: 20% Obese: 10%%
Time since diabetes diagnosis	3 months [4 days to 12 months]
Glycemia	295.45 ± 29.67 mg/dL
DM treatment	Caninsulin
Typical DM signs	Polydipsia: 90% Polyuria: 90% Polyphagia: 50% Weight loss: 90%

3.2. Owners' perception of digestive changes

Out of the ten owners that completed the anamnesis, five reported at least one digestive change in their diabetic animals, representing a prevalence of digestive alterations of 50%. These alterations were either gastric or intestinal changes, but three dogs experienced both. Regarding the upper GI tract, three owners indicated that their dogs went from not vomiting to consistently vomiting either around the time of diabetes diagnosis or afterward, with a related frequency that ranged from one to four times *per* week. Two of these owners noted that vomiting typically occurred within 15 to 30 minutes after a meal.

In terms of defecation habits, three owners reported an increase in defecation frequency, while two reported an increase in stool volume. Only one owner reported constipation, with a decrease in defecation frequency. Diarrhea was described in two animals. One dog also presented a behavioral change regarding defecation, starting to defecate inside the house followed by coprophagia.

Concerning fecal appearance, in addition to increased volume, owners reported various changes such as stronger odor (two dogs), watery feces and thicker stools (one dog).

3.3. Ultrasound evaluation of the GI tract

Compared to other species, the variability in dog breeds is remarkable, with extreme differences in size and weight. Regarding GI ultrasonography, body weight appears to have a greater impact on duodenal and jejunal wall thickness, while its effect on stomach and colonic wall thickness is less pronounced[29]. However, there is still some conflicting information on the intervale categories used for the different weights. The most widely accepted classification is that of Gladwin *et al.*, which categorizes dogs into three groups

based on body weight: less than 15 kg, 15–30 kg, and more than 30 kg[28]. This classification was used in the present study. All values were compared to the maximum reference values (RV) reported in the literature[28,30,31], except for inter-rugal evaluations, as no reference values have been established for healthy dogs. Additionally, there is only one RV for ileum in a study with a range of different weights.

In dogs <15 kg (*n*=4), there was an increased thickness of the gastric wall (6.00 ± 0.42 mm vs RV:3.3 mm[30]) with an inter-rugal value of 3.16 ± 0.45 mm. This increased in the thickness of the GI wall was also observed in the duodenum (5.19 ± 0.31 vs RV: 3.8 ± 0.5 mm[28]) and jejunum (3.84 ± 0.25 vs RV: 3 ± 0.5 mm[28]). On the other hand, the ileum and colon walls displayed normal thickness in diabetic dogs (ileum: 2.81 ± 0.15 mm vs RV: 4.83 ± 1.22mm[31]; colon: 2.03 ± 0.23 vs RV: 1.5 ± 0.03 mm[28]) (figure 1).

In dogs between 15 to 30 kg (*n*=3), there was an increased thickness of the gastric wall (6.27 ± 1.04 mm vs RV:3.25 mm[30]) with an inter-rugal value of 3.98 ± 0.24 mm. This increased in the thickness of the GI wall was also observed in the jejunum (4.49 ± 0.34 vs RV: 3.5 ± 0.5 mm[28]). On the other hand, duodenum, ileum and colon walls displayed normal thickness in diabetic these dogs (duodenum: 4.68 ± 0.18 vs RV: 4.1 ± 0.7 mm[28]; ileum: 3.76 ± 0.63 mm vs RV: 4.83 ± 1.22mm[31]; colon: 2.09 ± 0.48 vs RV: 1.4 ± 0.5 mm[28]) (figure 1).

In dogs over 30 kg (n=3), there was an increased thickness of the GI wall in the duodenum (5.33 ± 0.53 vs RV: 4.4 ± 0.7 mm[28]) and jejunum (4.50 ± 0.44 vs RV: 3.7 ± 0.4 mm[28]). On the other hand, stomach, ileum and colon walls displayed normal thickness in diabetic these dogs (stomach: 5.10 ± 0.72 vs RV:5 mm[30]; ileum: 4.93 mm vs RV: 4.83 ± 1.22mm[31], n=1; colon: 2.31 ± 0.25 vs RV: 1.6 ± 0.4 mm[28]) (figure 1).

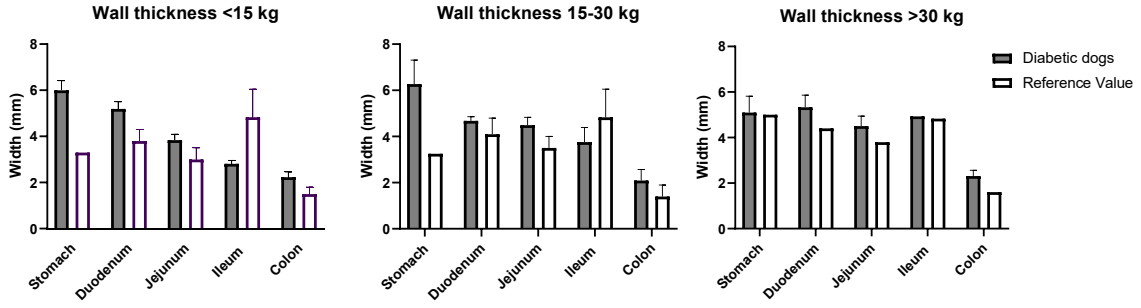

Figure 1 - Total wall thickness (mm) of the stomach, duodenum, jejunum, ileum, and colon measured using ultrasound in diabetic dogs <15kg (n=4), 15-30kg (n=3) and >30kg (n=3) compared to reference values previously documented in the literature.

Regarding the stomach, ultrasound measurements of the gastric wall at the level of the rugal fold ranged from 5.32 to 6.76 mm (<15kg), 5.23 to 7.32 mm (15-30kg) and 4.38 to 5.82 (>30kg) (figure 2A). Only one dog had average values within the normal reference

mean (maximum of 5 mm), while all the others presented values above the reference range. Three diabetic dogs showed an average of the three measures above 6 mm, which is typically considered pathological[32].

In duodenum, values ranged from 4.48 to 5.96 mm (<15kg), 4.5 to 4.87 (15-30kg) and 4.6 to 6.36mm (>30kg) (figure 2B). Most dogs presented values above the RV and one of the animals presented a value above 6 mm. The same was true for the jejunum, where values ranged from 3.58 to 4.32 mm (<15kg), 4.16 to 4.83 mm (15-30kg) and 3.62 to 4.96 (>30kg) (figure 2C).

The ileum was not easily visualized in all animals, especially in the group >30kg, meaning that it was not evaluated in all the diabetic dogs. The veterinary imagiologist was able to confidently measure the ileum in 6 dogs, with values ranging from 2.66 to 4.93mm, all within the RV (figure 2D).

According to *Gladwin et al.*, colon thickness does no vary according to the animals' weight[28]. Although the average colon measurements did not differ significantly from the normal reference values, most of the dogs had values above 2 mm (1.74 to 2.86 mm) (figure 2D).

In a normal GI ultrasound, five echogenic layers are identified: the innermost hyperechoic layer corresponds to the surface of the mucosa; the inner hypoechoic layer represents the mucosa; the middle hyperechoic layer is the submucosa; the outer hypoechoic layer is the muscularis propria; and the outermost hyperechoic layer is the subserosa/serosa[30]. Although sometimes it was possible to observe the increase of wall thickness, in all ultrasound examinations the normal GI mural stratification was preserved, making it possible for us to identify the layers previously described.

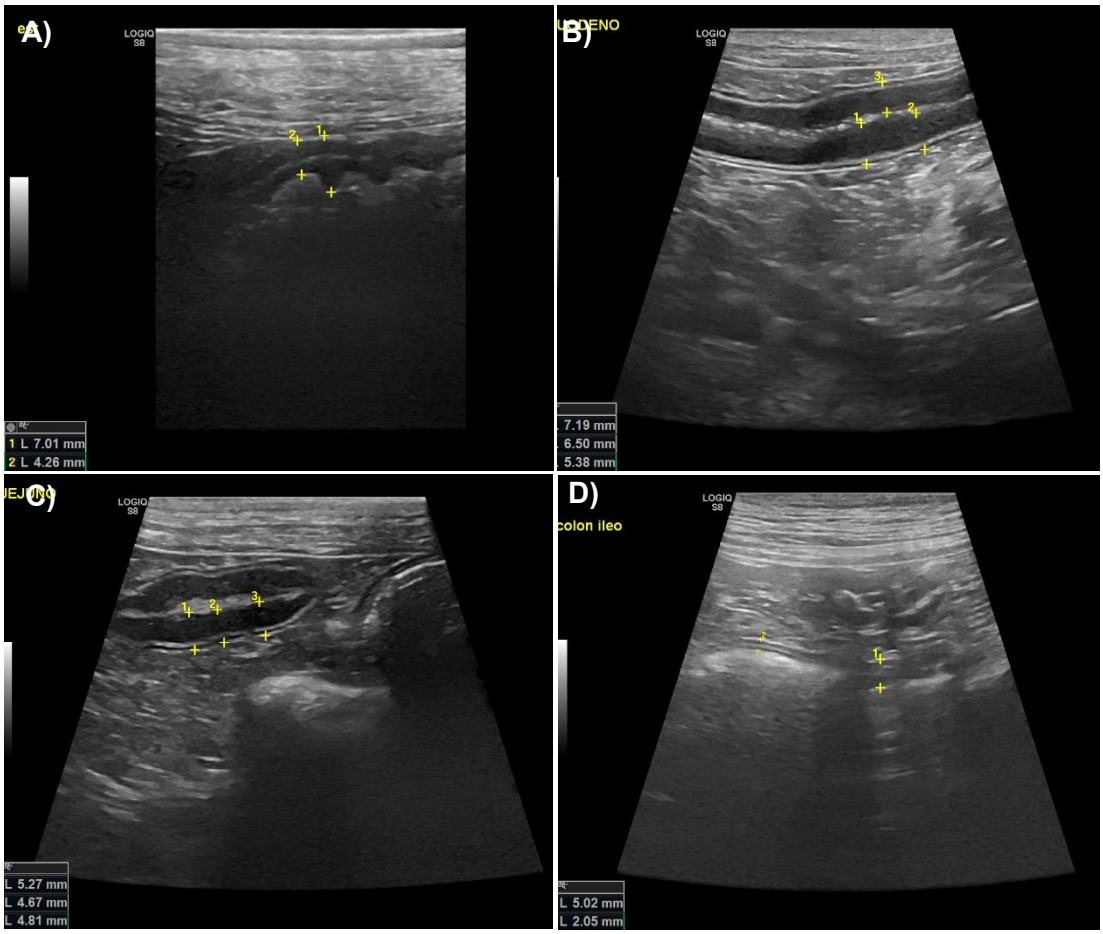

Figure 2 - Representative ultrasound images of the stomach (rugal fold and interrrugal - A), duodenum (B), jejunum (C) and ileo-colic transition (D), with measurement of both colon and ileum of diabetic dogs (longitudinal plans, using a 9-11 MHz probe). In these images all portions except ileum and colon present wall thickening.

3.4. Necropsy and histopathological evaluation

This animal was receiving treatment at UP-VET and developed uncontrolled diabetic ketoacidosis, which ultimately led to its death. The body was then donated to the study. It was a 6.6 kg, non-spayed, mixed-breed female with a six-month history of diabetes. Despite being treated with PROZinc at 17 IU - considered a high dose - it showed poor response to therapy. The dog had no prior history of gastrointestinal issues until its final presentation, during which it exhibited vomiting and hyporexia.

During necropsy, we observed that the intestinal segments had a rubber-like texture and were significantly thickened. Upon opening the segments, the walls remained rigid and retained their structure, preventing complete opening. Additionally, multiple areas of hyperemia were noted in the mucosa, characterized by a pronounced reddening of the tissue.

Histopathological evaluation revealed the presence of inflammatory infiltrates (figure 3A) throughout the entire gastrointestinal tract, with varying degrees of intensity, although they were consistently observed in all segments. Masson's trichrome staining was positive in the muscular layers (Figure 3B), indicating the deposition of collagen, which suggests the development of fibrosis. We performed measurments of the indivudal layers of the GI wall, however without control groups or reference values for histopathological measurements, we cannot definitively conclude the existence of thickening.

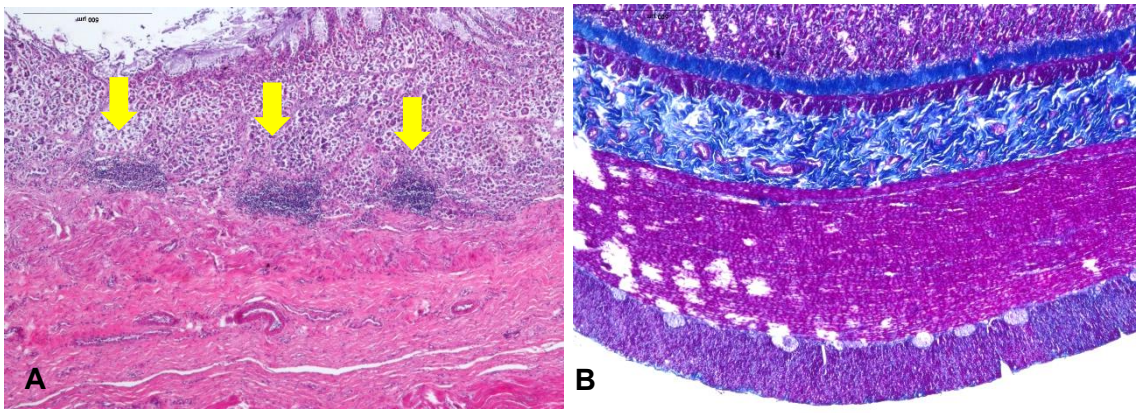

Figure 3 – A) Representative microscopic photograph stained with hematoxylin and eosin. Inflammatory infiltrates (yellow arrow) are observed on stomach of a diabetic dog. B) Representative microscopic photograph stained with Masson's Trichrome of fibrosis in diabetic jejunum. All images were captured using 40× magnification.

4. Discussion

This preliminary study represents the first in-depth investigation into the gastrointestinal health of diabetic dogs, revealing compelling similarities to human diabetes. The results indicate that diabetic dogs may exhibit symptoms akin to those seen in humans, with 50% of dog owners reporting noticeable digestive changes. Ultrasound assessments revealed significant thickening of the GI wall, while histopathological analysis of one diabetic dog revealed extensive fibrosis and inflammatory infiltrates throughout the gastrointestinal tract. The demographic data regarding the dogs included in this study partially aligns with our expectations. Most cases of spontaneous diabetes occur in middle-aged dogs (7-12 years)[2] and the average age of the dogs that underwent ultrasound falls within this range, as well the necropsied diabetic dog. However, literature states that intact female dogs are affected twice as often as males[8], and in this study most subjects were male. Most of the dogs in this study were purebred, thus reinforcing the possible genetic predisposition for diabetes. However, none of the breeds in this study correspond to those known to have a genetic predisposition to diabetes[5,7]. In fact, it is curious that the most common breed observed was

the Labrador Retriever, while the Golden Retriever is known to have a reduced risk of developing diabetes[8].

In the group of dogs included in this study, the animal that had been diagnosed with diabetes the longest met the average survival rate described in the literature. It appears that a percentage of diabetic dogs are euthanized at the time of diagnosis, while another group is euthanized within a year. Additionally, deaths related to poor glycemic control (especially important in T1D) and diabetes complications contribute to a shortened lifespan, thus decreasing the time of life after diabetes diagnosis[9].

It was not surprising that the most common gastrointestinal sign observed in diabetic dogs were vomiting and increased defecation frequency/diarrhea. However, only half of the dogs in this study exhibited such changes, while GI complications of diabetes appear to affect up to 75% of diabetic human patients[16,17]. Some of the most common GI symptoms of diabetes in human population are vomiting (mostly due to gastroparesis), constipation, diarrhea, and fecal incontinence[18,33]. Even in humans, it is not entirely clear whether these complications depend on the type of diabetes[16]. T1D accounts for only a small percentage of all diabetes cases (approximately 5%)[10], so the majority of clinical cases involve T2D patients. Since dogs only experience T1D, we cannot directly compare the prevalence of gastrointestinal complications in dogs with the general prevalence in humans.

Additionally, the manifestations of these complications are associated with poor glycemic control[34]. In general, the dogs in this study maintained glycemic levels within the expected range for diabetic patients, which can help explain why only half presented signs of gastrointestinal alterations. In general, our results agree with the only bibliographic reference on this subject, a review of cases of diabetes from the Colorado State University Veterinary Teaching Hospital, where about 38% of those also had GI disease[25].

Vomit was the most common symptom described in the diabetic dogs of this study. In fact, gastroparesis in human diabetic patients is well-studied, but still poorly understood[18]. Poor glycemic control is thought to disrupt gastric coordination and emptying, with neuronal damage[35] and gastric wall[36] remodeling as identified risk factors. There is only one paper focused on the GI tract of diabetic dogs that unveiled a notable reduction in the density of nitrergic neurons in both the antrum and ileum of diabetic dogs compared to the control group. Additionally, diabetic dogs exhibited a thickening of the periganglionic connective tissue surrounding the ganglia. The study concluded that diabetes in dogs leads to significant alterations in the myenteric neuronal composition, particularly affecting the nitrergic neuronal subpopulation[26]. This kind of neuronal damage has also been linked to gastroparesis and vomiting in humans[37].

Diarrhea and increased defecation frequency were the second most reported sign. Diarrhea in diabetic patients is multifactorial, often linked to advanced glycation end-products,

neuronal damage, and intestinal wall remodeling, especially due to muscular layers fibrosis[22]. It is typically intermittent, watery, and painless, and can be associated with fecal incontinence in a third of patients[38,39].

Only one dog owner reported that their dog began defecating inside the house, which was unusual. This behavioral change may be linked to fecal incontinence. In humans, this symptom is often caused by anal sphincter dysfunction and neuronal damage, possibly worsened by acute hyperglycemic episodes[40], which can explain why only one dog presented this symptom. This issue can create significant challenges for pet owners, adding both time and financial burdens for cleaning and repairing damage, which may strain the bond between owner and pet[41,42], a serious problem that is frequently cause for abandonment or euthanasia[43]. Given that only one dog in this study displayed such behavior, we suggest distributing a general questionnaire to diabetic dog owners, to determine if this is a common issue.

Given that half of the owners reported digestive alterations in diabetic dogs, it raises the question of whether corresponding morphological changes occur in the GI tract. Ultrasonography, a pivotal diagnostic tool[44], revealed a general thickening of the jejunum, duodenum, and stomach in most of the dogs, with some animals exhibiting gastric and duodenum wall thickness exceeding 6 mm. Histopathological analysis of one diabetic dog demonstrated gastrointestinal collagen deposition (indicative of fibrosis) and the presence of inflammatory infiltrates.

Prior research has established that the thickening of stomach muscle layers, driven by collagen deposition, is a hallmark of diabetes in both human and experimental models[45,46]. This thickening induces gastric wall stiffness, impairs absorption, and disrupts motility, potentially leading to abnormal gastric emptying[37], which may explain the increased vomiting frequency observed in diabetic dogs. Moreover, the altered gastric motility is also important as it can contribute to erratic glycemic control, with alternating episodes of hyperglycemia and hypoglycemia[47]. The intestinal thickening in diabetic dogs' mirrors findings in other studies, where increased thickness is linked to several factors such as accumulation of Advanced Glycation End Products and collagen type I, leading to gut stiffness, reduced compliance, and potential dysbiosis[22,48,49]. These changes, coupled with smooth muscle cell hypertrophy[50], likely contribute to motility disturbances and symptoms such as diarrhea[23,51], another GI complication observed in the diabetic dogs included in this study.

Considering the histopathological findings, the first observation was that the inflammatory infiltrates found throughout the GI tract were not indicative of inflammatory bowel disease. This finding aligns with the chronic low-grade inflammation commonly observed in diabetic patients, particularly those with T2D[52], where systemic inflammation and elevated cytokine

levels disrupt cellular functions and promote GI inflammation[53–55]. Diabetes-induced alterations in gut microbiota[53] and increased intestinal permeability ("leaky gut") further exacerbate inflammation, contributing to fibrosis and impaired GI function[56], thereby worsening the clinical manifestations of diabetes.

Taken together, data presented in this study provides preliminary evidence suggesting that diabetic dogs experience GI manifestations similar to those observed in humans. Symptoms such as vomiting and diarrhea, GI thickening observed in abdominal ultrasound, and fibrosis and inflammatory infiltrates identified in histopathological examination were noted. However, these results are limited due to the small sample size. The power analysis for this study indicated that at least 11 dogs *per* weight group were required, yet only 3 to 4 animals *per* group were available for examination. Additionally, histopathological analysis was conducted on a single dog, further limiting the scope of the findings. While these results suggest that diabetic dogs may indeed exhibit similar gastrointestinal complications, further studies with larger sample sizes are needed to confirm and validate these findings.

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Chapter 4 – General Discussion and Concluding Remarks

General Discussion

Diabetes is a complex, chronic, and progressive metabolic disorder that is medically incurable and can impact almost every organ system[1]. The prevalence of diabetes is alarmingly high across all age groups worldwide, with incidence rates continuing to rise annually[2]. GI complications are particularly common in diabetic patients, affecting up to 75% of this population and significantly diminishing their quality of life[3]. Despite the high prevalence of these complications, there is still a significant lack of comprehensive understanding and awareness of their importance[4,5], leading to a gap in their effective recognition and treatment[6].

Currently, treatment options for GI complications in diabetes are predominantly symptomatic, as no curative or preventive therapies have been established. For gastroparesis, prokinetic agents are commonly used to enhance gastric motility and accelerate stomach emptying[3]. However, these treatments are often ineffective, leading to the use of endoscopic or surgical interventions that are far more invasive. For instance, endoscopic pyloric injections of botulinum toxin have shown to improve symptoms and accelerate gastric emptying, but the effects only last up to 3-6 months[7]. Only completion gastrectomy seems to provide long-term symptom relief in some patients with gastroparesis, though data on its effectiveness in diabetic gastroparesis remain limited[8]. The management of diabetic diarrhea focuses primarily on symptom relief, correction of fluid and electrolyte imbalances, improvement of nutrition and glycemic control[3]. Loperamide may be useful in managing fecal incontinence, while severe constipation may require treatments such as lactulose and osmotic laxatives. There are some newer medications for chronic constipation, such as prucalopride and lubiprostone, but it doesn't seem to effectively solve these symptoms[3,9]. It is important to note that these treatments are only symptomatic, meaning they do not prevent or reverse the progression of GI alterations. Consequently, patients continue to suffer from these evolving complications, which contribute to a decline in their quality of life.

The RAAS is primarily recognized for its role in the cardiovascular and renal systems, regulating blood pressure and fluid homeostasis. However, RAAS activation is also implicated in diabetes, as ACEIs or ARBs have been shown to reduce the incidence of vascular complications, nephropathy, and cardiovascular disease in diabetic patients[10]. In patients with type 2 diabetes, RAS inhibition improves insulin sensitivity and better glycemic control[11] and infusion of Angiotensin II has been linked to insulin resistance[12]. However, there is currently no information regarding the potential of RAAS manipulation in preventing or treating GI alterations associated with diabetes.

And what about diabetic pets? Diabetes is also a significant concern for cats and dogs, with its prevalence on the rise[13]. Despite the importance of GI alterations in the human diabetic population, little is known about whether similar complications exist in diabetic pets[14]. These complications could also impact companion animals, with their discomfort potentially going unnoticed and untreated. Moreover, studying these alterations in diabetic animals will not only improve their well-being but could also provide valuable insights for human medicine, as spontaneous diabetic cats serve as optimal models for type 2 diabetes[15], while spontaneous diabetic dogs can be used as models for type 1 diabetes[16].

Given these considerations, this thesis has two major objectives. The first is to demonstrate diabetes-related gastrointestinal complications in experimental models of both type 1 and type 2 diabetes and to experimentally test ARBs as a preventive measure for these alterations. The second objective is to conduct clinical studies in spontaneously diabetic dogs and cats, using GI-focused anamnesis, abdominal ultrasound to evaluate GI alterations, and *post-mortem* evaluations of diabetic pets donated for necropsy.

To address these objectives, this study explored five major research questions:

1. **How do the macro and microscopic structures of the GI tract change in both T1D and T2D rat models and which cell types are affected? Do the intestines of diabetic rats exhibit functional changes?**

To address this question, a short-term model of T1D and a long-term model of non-obese T2D were used. The STZ model of diabetes is well-established for mimicking the effects of T1D when administered as a single high-dose IP or IV injection[17]. This method induces β-cell death, resulting in an inability to produce insulin and leading to the typical complications associated with diabetes[18]. Although most studies examining GI complications in diabetes have employed longer protocols[19], we chose a short-term model. This decision was supported by a previous study that observed significant changes in the intestinal wall as early as one week after diabetes induction[20]. With animal welfare as a priority, we opted to evaluate the GI tract at two weeks post-induction.

GK rats were used as the non-obese T2D model, which effectively mimic diabetes-related complications as the animals reach adulthood. We studied animals at 21-22 weeks of age (147-154 days), a period considered to represent long-term diabetes. While it could have been insightful to use the STZ model to study T2D as well, the GK model was specifically chosen to eliminate obesity as a confounding factor [21]. By avoiding the variable of increased body size, which could correlate with a larger GI tract [22], we aimed to focus on the direct effects of diabetes on GI structure.

As expected, both models exhibited typical diabetes signs, including polydipsia, polyuria, and polyphagia. The STZ-induced animals showed noticeable weight loss, while the GK animals did not. However, the GK rats consistently weighed less than their age-matched Wistar counterparts, which is consistent with the characteristics described for this model[23]. Both STZ-induced and GK rats also exhibited hyperglycemia, a hallmark characteristic of DM. In the STZ-induced group, hyperglycemia was attributable to the cytotoxic effects of STZ on pancreatic β-cells, leading to insulin deficiency. The persistent hyperglycemia observed in STZ-induced rats suggests progressive cellular damage, with blood glucose levels reaching up to four times higher than those of non-diabetic controls[20,24]. Interestingly, GK rats displayed blood glucose levels approximately half that of STZ-induced rats, while still remaining hyperglycemic. This difference is expected when considering the distinct types of diabetes in the two groups. In contrast to the STZ-induced model, where insulin production is absent[17], GK rats primarily exhibit reduced pancreatic β-cell function and peripheral insulin resistance[25]. Therefore, it is anticipated that these animals would present with moderate hyperglycemia[23].

Although the data on typical diabetes signs and hyperglycemia may not directly address the question of this subchapter, it was crucial to unequivocally confirm that the animals in both experimental groups were representative of T1D and T2D.

Regarding the study of the macroscopic characteristics of the diabetic GI tract, we were only able to study STZ rats due to logistical constraints in collecting the GI tract from the GK animals. This evaluation started with fecal excretion and our study was the first one to quantify fecal excretion in diabetic models. We found that diabetic rats gradually increased their fecal excretion, reaching values 4 times higher than those obtained in the first day. Besides the increase in quantity, the fecal pellets also presented visual qualitative differences. The fecal pellets from the diabetic group were well formed but were bigger, larger and darker. Cuervas-Mon and collaborators also observed some differences in the feces of STZ-induced diabetic rats, but they described them as being thick and amorphous[26]. This finding could eventually be ascribed to polyphagia and the reported distension of the intestinal wall.

Compared to the control animals, all sections of the intestines in STZ rats appeared enlarged, consistent with observations previously reported by Cuervas-Mon and colleagues[26]. Upon opening the abdomen of STZ-induced rats, the distension of the intestines was immediately noticeable. Additionally, these animals exhibited an extremely dilated cecum, which created a “mass effect”, displacing the intestines to the side. Our data demonstrates that just two weeks after induction, STZ-induced rats already exhibit significant macroscopic intestinal alterations, including increased length, perimeter and weight, along with a greater weight of intestinal contents. Forrest *et al.* similarly observed a

significant increase in dry colon weight in diabetic animals compared to controls, attributing this to increased colon length, as weight *per* unit length did not differ between the two groups[27]. Other researchers also reported significantly higher weights in the colon and ileum of diabetic animals (whether treated with insulin or not) compared to controls[28,29]. Intestinal smooth muscle cells are known to be plastic and adapt to functional demands through remodeling[27]. Jervis *et al.* proposed that intestinal enlargement in diabetic animals is an adaptation to polyphagia[30]. In their study with alloxan-induced diabetic rats, they observed that abdominal distention was due to increased fecal content, along with an enlarged diameter and length of the small intestine and colon[30]. Interestingly, other conditions associated with polyphagia, such as lactation[31] or hypothalamic lesions[32], also induce GI enlargement. However, a study contradicting this hypothesis found that even when the food intake of diabetic rats was matched to that of controls, intestinal mass remained higher in diabetic animals[20]. This suggests that only part of the intestinal growth is related to food consumption. Other explanation is the increased expression of glucagon-like peptide 2 (GLP-2), an intestinal trophic hormone that promotes intestinal growth in diabetic animals by nearly doubling plasma concentrations of GLP-2 and enteroglucagon, leading to intestinal epithelial proliferation[33]. Additionally, it has been proposed that megacolon and ileum enlargement may be secondary to diabetic autonomic neuropathy (DAN), where a reduction in neuronal numbers could cause hyperplastic and hypertrophic changes in the intestinal walls[34].

The GI tract of both models of diabetes was evaluated histopathologically, with assessments conducted on the total thickness of the intestinal wall as well as the thickness of each individual layer: longitudinal muscle, circular muscle, submucosa, and mucosa. In both cases, diabetic animals exhibited significant alterations in the thickness of the total intestinal wall and/or its individual layers, with variations depending on the specific intestinal section and the diabetes model.

To our knowledge, no prior histopathological data existed on the colon of STZ-induced rats just two weeks after induction. A previous study on STZ-induced rats reported similar findings to ours, but only in the ileum, where increased mucosa (the most significantly affected layer), submucosa, muscle layer, and total wall thickness were observed[20]. Several other studies have found increased thickness in various parts of the GI tract in diabetic animals, including the colon and ileum [35,36].

The observation in our first study that differences between diabetic and control animals become less pronounced in the distal direction aligns with the findings of Fregonesi *et al.* These authors demonstrated that diabetes affects the GI tract differentially, with the distal segments being affected last[37]. With this in mind, when studying the intestines and colon of GK animals. We chose to assess both the proximal and distal colon, as well as all segments

of the intestine duodenum, jejunum and ileum, to determine if a similar proximal-to-distal progression of the disease, as previously described in T1D models[37] and observed in our initial study, would be present. In this model, we did not observe such a pattern, as gut remodeling appears to occur in a more random manner, consistent with findings from other studies[38]. Could this difference be related to the type of diabetes? Existing data is unclear on whether there is a difference in the prevalence and severity of GI symptoms between T1D and T2D patients[39]. However, the prevalence of GI symptoms depends on the duration of the disease and the region affected in T2D[40]. Indeed, in these patients, intestinal changes were the first symptoms to appear, with constipation and diarrhea occurring with equal prevalence but at different times, while gastric alterations were observed later. Notably, only the scores for constipation and diarrhea showed bimodal peaks, the first in the early stages of diabetes and the second later in the disease course[40]. This clinical study in T2D patients infers that these alterations do not follow a proximal-to-distal progression, consistent with the findings of our study.

The increase in both the mucosa and muscle layers of the intestine in diabetic conditions is a multifaceted process influenced by several mechanisms[19]. As stated before, one primary factor contributing to the increased thickness of the intestinal wall is food consumption[37]; however, this is not the only mechanism at play. Suppression of apoptosis in the intestinal mucosa appears to play a crucial role, particularly in the early stages following STZ injection, where apoptosis decreases before returning to normal levels after three weeks. This transient suppression, combined with a gradual increase in food intake, may significantly contribute to the initial enlargement of the intestinal mucosa in STZ rats[41]. GLP-2 has been shown to exert a trophic effect on the intestinal epithelium, an effect that is enhanced in diabetic animals[33,42]. Additionally, GLP-2 inhibits apoptosis in the small intestine, further contributing to its enlargement[43]. Additionally, in GK rats, intestinal hyperplasia has been linked to increased expression of transcription factors and proteins involved in cell regeneration, differentiation, and proliferation[44].

The muscular layers of the diabetic intestine also undergo significant changes. Prior research has documented extensive remodeling in the diabetic gut, characterized by increased production and accumulation of collagen type I and AGE[19,36,45–47]. This accumulation primarily occurs around and between smooth muscle cells (SMCs), leading to stiffening of the intestine and reduced resting compliance (Siegman *et al*., 2016). Additionally, the existence of hypertrophy of the SMCs within the muscular layers of the diabetic GI tract has been previously described[48,49], a finding that our results also corroborate. This hypertrophy, which involves an increased number of contractile proteins like actin and myosin[48], is consistent across various portions of the gut.

In summary, the thickening of the diabetic intestinal wall appears to be driven by both mucosal proliferation - due to factors such as increased food intake, enhanced GLP-2 expression, and suppression of apoptosis, and the hypertrophy of muscle layers - attributed mostly to AGE accumulation, SMCs enlargement and collagen type I accumulation. These changes collectively contribute to the altered structure and function of the diabetic intestine. It has been suggested that the increased thickness of GI tract may also be secondary to neuronal damage. A reduction in the number of neurons appears to induce hyperplastic and hypertrophic changes in the intestinal walls [34], presenting new avenues for research. Given the critical role of the myenteric plexus in controlling GI motility, we chose to quantify the neuronal population within this plexus[24]. Our work and other several authors reported changes in the number and/or size of myenteric neurons through the entire GI tract, including both T1D[50] and T2D[51] models. Several mechanisms have been proposed to contribute to neuronal loss, including increased apoptosis, elevated levels of AGEs and their receptors, reduced nerve growth factor levels, and oxidative stress[52,53]. The altered number of neurons in the GI tract is associated with the typical gastrointestinal symptoms reported by diabetic patients[54]. This neuronal change can lead to improper gut motility, retrograde colonic movements, altered secretions, and even increased pain stimuli[55,56].

One approach to study gut function involves testing the response to various neurotransmitters known to influence both the motility and central regulation of the gut. In our experimental work, the contractile response to ACh and KCl between control and STZ animals had similar E_{max} and EC_{50} values. A prior study found that cholinergic nerve expression and acetylcholinesterase histochemistry produced comparable results in both diabetic and control animals[26]. Other studies assessing the colonic response to exogenous ACh found no differences between control rats and those with STZ-induced diabetes 30 days after induction[57]. Only after 60 weeks of disease progression in a genetic model of diabetes, the proximal colon showed a diminished contractile response to carbachol, an acetylcholine analogue, compared to controls, whereas the distal colon's response remained unchanged[58]. We could infer that cholinergic activity in the GI tract may vary with the duration of diabetes but even after an extended period of illness (6 months post-STZ induction in rats), the response to exogenous ACh was maintained [59].

Given the significance of the RAAS in this thesis and the contractile effect of Ang II in the gut, we decided to test the reactivity to Ang II by generating a non-cumulative response curve using different concentrations of exogenous Ang II. This study marks the first time that the reactivity to Ang II has been investigated in the intestine and colon of diabetic animals. The findings of this functional study suggest a loss of contractile force in the ileum, proximal colon and middle colon — though not in the distal colon — of STZ-induced rats in response to Ang II. In the rat colon, Ang II receptors are predominantly located in the SMCs

of the muscularis, with a smaller presence in the mucosa[60]. It was previously described that AT_1R and AT_2R were also located on the myenteric nerves in human colon while in the guinea pig they only found AT_1R [61,62]. The predominant receptor is AT_1, but a small number of AT_2R were also observed [63]. Given the distribution of Ang II receptors, the altered response in STZ-induced rats may be related to structural changes such as neuron loss, particularly in the myenteric plexus[34]. However, increased local tissue levels of Ang II could also account for the decreased reactivity and the observed plateau at lower concentrations, potentially due to receptor desensitization as previously described[64]. To rule out the possibility that receptor desensitization was due to the experimental protocol, we washed the tissues for 1 hour between each concentration of Ang II tested and discontinued the concentration-response curve when two consecutive responses were similar or when a higher concentration produced a lower response than the previous one. The maximum response in control animals was similar to that found in another study, where they described the concentration close to log 10^{-7} M as being responsible for the maximum effect on the colon[61] and small intestine[64].

In this protocol, as expected, Ang II was always able to induce contractile responses mediated by AT_1R activation[65,66]. Regarding AT_2R activation, these receptors are known to counterbalance Ang II-mediated AT_1R actions[62], an effect that was observed in colonic segments of control rats. However, in the ileum, middle colon, and distal colon of STZ-induced rats, antagonism of AT_2R with PD123319 had no effect on Ang II-mediated contraction. This suggests that, in these regions, AT_2R do not functionally counteract AT_1 receptor-mediated contraction, as previously described in an experimental model of colitis in rats[67]. Interestingly, on the PC of STZ rats and on the ileum of control rats AT_2R seems to behave differently than the remaining portions, since the contractile effect of Ang II decreased in the presence of PD123319. The AT_2R-mediated contractile effect is not common but has previously been described in a vascular study[68]. Some researchers propose that the distribution and function of Ang II receptors might alter in the presence of gastrointestinal disorders and other pathophysiological conditions[64,69,70]. However, further investigation is needed to fully understand how these changes impact Ang II responses.

When employing animal models, adherence to the 3Rs (Replacement, Reduction, and Refinement) is paramount, and these principles are integrated into all our experimental protocols[71]. The STZ-induced diabetes protocol was carefully refined to minimize fasting duration prior to induction, alongside the incorporation of analgesia. This modification was implemented based on the rationale that the β-cell apoptosis induced by STZ may involve nociceptive processes, although this remains uncertain. This was the first protocol to integrate analgesia, and this intervention did not compromise the experimental outcomes in

the two instances in which it was applied. Furthermore, we opted for a short-term diabetes model rather than maintaining animals in a prolonged diabetic state, thus further contributing to the refinement of our approach.

Additionally, in the interest of reducing animal use, we utilized GK rats in conjunction with other experimental groups. Once the other groups had completed their required interventions, they generously provided us with the gastrointestinal tracts, significantly reducing the number of animals required for the study (Reduction). In the STZ protocol outlined in Paper 2.4, we also implemented organ-sharing strategies, facilitating the use of tissues such as brain and kidneys, which were subsequently shared with collaborating research teams. We also collected additional biological samples from the same cohort of animals for further investigation.

2. Is the gastrointestinal tract altered in diabetic cats and dogs? Do diabetic pets exhibit gastrointestinal clinical signs?

Reports of GI manifestations in humans associated with diabetes date back to at least 1971[72]. However, in 1995, Diehl highlighted a significant gap in our understanding of these manifestations in diabetic cats and dogs[14]. Despite the rising prevalence of diabetes in pets and their increasing life expectancy[13], research and documented cases on the GI health of diabetic pets remain surprisingly scarce. This raises a critical question: are diabetic cats and dogs genuinely free from GI complications, or are these issues simply underdiagnosed and untreated, similar to the challenges faced in human patients[6]? Based on our preliminary findings, the short answer is that diabetic cats and dogs do indeed exhibit GI manifestations of diabetes. These manifestations were identified through multiple avenues: owners reported noticeable digestive changes in their pets, ultrasound imaging revealed increased GI wall thickness in diabetic animals, and histopathological evaluations confirmed widespread alterations across the GI tract. Specifically, we observed significant thickening of the muscular layers, the presence of inflammatory infiltrates throughout the GI tract, and fibrosis.

A majority of diabetic cat owners reported noticing at least one digestive change in their pets (more than 80%), whereas only about 50% of diabetic dog owners observed gastrointestinal changes in their dogs. The percentage of cats exhibiting GI symptoms related to diabetes closely aligns with observations in human patients, where approximately 75% are affected[4]. It is known that cats and dogs undergo different mechanisms when developing diabetes and exhibit different types of the disease[73,74]. Most diabetic cats experience a condition similar to human T2DM[75], while all diabetic dogs have an insulin-

dependent form akin to human T1D[16]. Additionally, most diabetes cases in humans are T2D, which accounts for 90 to 95% of all diabetes cases worldwide[76]. Given this information, we might argue that most data on the prevalence of GI manifestations of diabetes comes from patients with T2D. This could reinforce the resemblance of feline diabetes and T2D in humans. However, there is currently no information indicating that the prevalence of GI symptoms varies based on the type of diabetes[39]. Thus, this remains a speculative suggestion.

Not surprisingly, the most frequently reported digestive issues in diabetic pets were vomiting and diarrhea, which are also common symptoms in diabetic human patients[3,77]. Constipation, while a common issue in diabetic individuals[3], was noted by only one owner. This may be due to the difficulty in detecting constipation in cats by their owners. Interestingly, one owner reported that their cat initially experienced obstipation in the early months of diabetes, which later progressed to diarrhea. This observation aligns with the common occurrence of constipation alternating with diarrhea in diabetic human patients[40,78]. Episodes of fecal incontinence can also occur in diabetic patients and are likely underdiagnosed. This symptom is often considered troublesome and may only be reported when patients are specifically questioned about it[78]. Regarding the cats in this study, reports from almost half of the owners about their pets defecating outside the litter box could indicate that these animals are experiencing episodes of fecal incontinence[79]. On the other hand, none of the dog owners reported similar behavior. It appears that these episodes are directly correlated to acute hyperglycemic episodes and poor glycemic control[77,78]. Therefore, the uncontrolled glycemia observed in cats in this study, which was not seen in dogs, may have contributed to this issue being exclusive to diabetic cats.

Most of the owners reported an increased stool volume. This observation aligns with our earlier results described in the previous subsection, where diabetic rats also exhibited increased fecal excretion. We attribute this finding to the polyphagia observed in diabetic animals[80], as there are no other reports of similar occurrences.

The next step was to assess whether there are morphological changes in the GI tract of diabetic cats and dogs. Ultrasound evaluations revealed that, while the integrity of the intestinal layers is maintained, there is noticeable thickening in the stomach, duodenum and jejunum. In some animals, the gastric wall thickness exceeded 6 mm which is typically considered pathological[81–83]. Additionally, histopathological evaluation of the GI tract in diabetic cats revealed increased thickness of the muscular layers across all studied regions, along with inflammatory infiltrates and fibrosis. Although only one diabetic dog underwent necropsy, the examination of its GI tract also showed inflammatory infiltrates and fibrosis. These results closely mirror our findings on laboratory animals, as discussed previously. Moreover, when looking at the GI wall total thickness in diabetic pets, it also appears to be

a proximal-to-distal progression, with the proximal segments of the GI tract, particularly the stomach, being the most affected. Increasing the thickness of the mucosal layer is significant because it can negatively impact nutrient absorption and potentially affect glycemic control[19,84]. The increased thickness of the muscular layers, partly attributed to collagen deposition around smooth muscle cells - as indicated by the positive Masson's trichrome staining - is a significant alteration. This change may help explain the GI clinical signs observed in diabetic cats. In fact, fibrosis in the diabetic gastrointestinal tract is linked to a loss of matrix elasticity and contractility, impairing both contraction and relaxation responses, which are crucial for maintaining normal GI motility[85,86]. These alterations are typically associated with neuronal damage and oxidative stress in the gastrointestinal tract[85,87]. However, we have not yet evaluated these factors in the GI tract of diabetic pets.

3. Is the expression of local RAAS enzymes (ACE, ACE2) and of the effector peptide (Ang II) altered in the gut of diabetic animals?

The RAAS is a fundamental regulatory network involved in cardiovascular, renal, and metabolic homeostasis[88]. The balance between ACE and ACE2 is essential for maintaining physiological homeostasis, and dysregulation of this system is implicated in various pathophysiological conditions, including diabetes[89]. Additionally, the local activity of the RAAS allows for fine-tuned control of tissue microenvironments, particularly in organs vulnerable to hypertensive, fibrotic and inflammatory damage[90].

In diabetes, systemic alterations in RAAS enzyme expression are well known, contributing to disease progression and complications[89]. In this study, we began by confirming that the systemic activity of ACE and ACE2 was elevated in diabetic animals, as previously described and verified. In fact, hyperglycemia is known to upregulate ACE expression, leading to increased Ang II production, oxidative stress, and endothelial dysfunction[12,89]. The increase in ACE2 may act as a compensatory mechanism[91], although it seems insufficient, as diabetic patients tend to have elevated circulating levels of Ang II[10].

Conversely, the local expression of these enzymes does not always align with the systemic alterations. Local ACE2 expression is often downregulated in diabetic conditions, exacerbating the local harmful effects of Ang II[92]. Reduced ACE2 activity leads to an imbalance in the RAAS axis, favoring vasoconstriction, fibrosis, and pro-inflammatory signaling[93]. For example, diabetic kidney disease is significantly influenced by the overexpression of ACE and downregulation of ACE2 in renal tissues, that contribute to glomerular hypertension, fibrosis, and albuminuria[94]. Keeping this in mind, we innovatively decided to explore the activities of ACE and ACE2 in the diabetic gastrointestinal tract.

The RAAS is not only critical for cardiovascular and renal function but also plays an essential role in gut physiology. These enzymes influence gut homeostasis, amino acid absorption, microbial balance, and inflammatory responses[69]. Both ACE and ACE2 enzymatic activities have been detected by our own group in the intestinal contents along the gastrointestinal tract, highlighting their role in local RAAS regulation[95]. This study marks the first observation of altered ACE activity, ACE's N/C-domains Ratio and ACE2/ACE ratio in the diabetic GI tract. Also, a notable discrepancy emerged between systemic and GI local RAAS activity in the diabetic state. While both systemic ACE and ACE2 levels were upregulated, resulting in a ACE2/ACE ratio comparable between experimental groups, local ACE2 activity within the GI tract remained unchanged, while ACE activity was significantly elevated resulting in a decreased ACE2/ACE ratio in some of the GI portions studied. This divergence aligns with the growing body of evidence suggesting that local, tissue-specific RAAS alterations contribute independently and heterogeneously to the pathophysiology of diabetic complications, distinct from systemic RAAS activity[96].

The study employed both Z-FHL and h-HL substrates to assess ACE activity, allowing analysis of the Z-FHL/h-HL ratio to reflect functional balance between ACE's N- and C-domains. The Z-FHL/h-HL hydrolysis rate ratio depends on the domain: both domains combined exhibit a ratio of approximately 1, the N-domain presents a ratio of 4.5, and the C-domain presents a ratio of 0.74[97]. Additionally, this ratio has been shown to vary across pathophysiological conditions and has potential as a biomarker for domain-specific shifts in ACE function[98]. The portions from the control group exhibited a Z-FHL/h-HL ratio notably greater than 4.5, which is indicative of a predominant N-domain activity. Also, previous studies in diabetic tissues indicate that increased activity in the ACE N-domain may account for tissue-specific remodeling[99]. Yet our observations in the diabetic gut indicate a maintained or a reduction in the Z-FHL/h-HL ratio in some portions (duodenum and jejunum). This reduction to values close to 2 shows a functional shift favoring enhanced C-domain activity - the primary domain responsible for Ang I to Ang II conversion. This C-domain-mediated rise in local ACE activity underscores the central role of Ang II in mediating GI tissue remodeling, including muscular hypertrophy and oxidative stress[100–102]. This supports previous findings that tissue-level Ang II actions, independent of systemic levels, drive local pathology[103]. From another perspective, this shift to a more balanced N/C domain activity could have a protective effect. The N-domain is known for the degradation of key bioactive peptides like Ac-SDKP[104], which has been shown to inhibit the activation of pro-fibrotic pathways and attenuate inflammatory responses that contribute to tissue remodeling[105]. Therefore, a decrease in the relative activity of N-domain activity could reduce the degradation of Ac-SDKP, thereby preserving its functions. This raises the question of whether an increase in ACE activity in diabetes, accompanied by a more

balanced interaction between the N and C domains, rather than a predominance of N-domain activity, might serve as a protective mechanism to preserve Ac-SDKP's anti-fibrotic and anti-inflammatory effects. However, this remains speculative.

Despite systemic ACE2 upregulation, unchanged local ACE2 activity in the GI tract indicates a decoupling between systemic and tissue-specific regulation. This dissociation may imply limited compensatory function by the ACE2/Ang 1-7/Mas receptor axis locally, shifting the RAS balance toward Ang II-dominant signaling. This imbalance is further emphasized by a reduced ACE2/ACE ratio, a phenomenon observed in other diabetic tissues such as kidney[106], retina[107] and pancreas[108], contributing to localized oxidative damage and inflammation[109]. Furthermore, the shift away from ACE2-mediated degradation of Ang II may be aggravated by increased ACE shedding, as previously described in renal tissues, which compromises local Ang II clearance[98].

Collectively, these findings support a paradigm where tissue-specific (but not systemic) RAAS dysregulation plays a pivotal role in diabetic gut pathology. In this context, therapeutic strategies targeting local RAAS components, such as ACE inhibition or AT_1 receptor blockade, may be considered as an option to mitigate these organ-specific complications at the tissue level.

4. Is the local activity of the glutathione system altered in the GI tract of both type 1 and type 2 rat models of diabetes?

Oxidative stress is closely linked to diabetes, its related complications[110] and to the RAAS detrimental effects[111]. In fact, ROS expression is one of the key mediators of Ang II-induced fibrosis[111,112]. Oxidative stress can be assessed through various methods, one of which is measuring the GSH/GSSG ratio. This ratio is a widely used indicator, as it typically decreases under oxidative stress. A lower ratio may reflect a shift toward a more oxidized state (increased GSSG), a depletion of GSH (due to reduced availability of the cofactor NADPH or GSH precursors), or both[113,114]. Additionally, total glutathione levels (the sum of GSH and GSSG) serve as another marker of oxidative stress, as a decline in its levels often points to impaired GSH synthesis or increased consumption due to excessive oxidative burden[115]. Extensive research has demonstrated the presence of oxidative stress in both diabetic patients and laboratory animals[110,116,117]. In DM, oxidative stress can result from hyperglycemia-induced metabolic changes, including aldose reductase activation and the formation of AGEs. It also seems to exist an impaired GSH recycling by glutathione peroxidase due to NADPH depletion[114].

While there is existing information about the GSH system in diabetic patients in several tissues and organs, the local GSH system within the diabetic GI tract was yet to be explored.

Addressing this gap, a part of this thesis focuses on characterizing the glutathione system across different GI tract sections in the experimental models of diabetes described earlier. As anticipated, the GSH/GSSG ratio decreased in all GI sections of diabetic animals. However, the mechanisms underlying this decrease varied according to the protocol used. In the short-term T1DM STZ-induced model, we observed an increase in tGSH, accompanied by a notable rise in GSSG levels. Conversely, in the long-term T2DM model, there was a reduction in both tGSH and GSH levels, while GSSG levels remained consistent between diabetic and control animals. Nevertheless, both scenarios indicate the presence of localized oxidative stress.

Although at first glance our findings may appear contradictory, they are consistent with the biphasic alterations in glutathione metabolism commonly observed in diabetes. In early-stage diabetes, systemic tGSH levels may initially rise due to enhanced precursor availability and increased synthesis as a compensatory response to oxidative stress[118]. However, this adaptive response is often accompanied by a concurrent rise in GSSG, leading to a decline in the GSH/GSSG ratio, a key indicator of redox imbalance[119], which we observed in our on short-term experimental diabetes model. This pattern likely reflects early compensatory antioxidant responses coupled with excessive ROS production, as described in previous studies[115]. The rise in GSSG levels has been shown to correlate with elevated ROS burden and increased glutathione utilization in both diabetic humans and animal models[120,121]. Over time, as chronic hyperglycemia persists, glutathione precursor depletion and impaired synthesis (partly due to reduced γ-glutamyl-cysteine synthetase activity) lead to a significant reduction in total GSH, further worsening redox imbalance[122]. Notably, in both short- and long-term diabetes models, we observed a roughly 50% reduction in the GSH/GSSG ratio in diabetic samples compared to controls. This finding aligns with previous reports describing similar redox shift in both intracellular and plasma compartments[120,123]. Importantly, this imbalance has also been implicated in impaired glucose metabolism and insulin sensitivity, suggesting that glutathione dysregulation is not only a marker but also a mediator of diabetic pathology[124].

5. Can targeting the intestinal RAAS using ARBs in diabetic rat models be a therapeutic approach to prevent diabetic gastrointestinal remodeling?

The findings presented in this thesis suggest that the use of ARBs in the context of diabetes may represent a promising therapeutic strategy to prevent gastrointestinal complications of diabetes. This constitutes one of the most relevant and innovative contributions of the present work. Notably, treatment with losartan was able to prevent muscular remodeling of the gastrointestinal wall in diabetic animals, while treatment with finerenone had no effect.

Furthermore, the reduction in oxidative stress observed - evidenced by the normalization of tGSH levels and GSH/GSSG ratios, approaching those of control animals - supports a protective redox-modulating effect. In addition, increased local ACE2 activity was detected in the jejunum and ileum of losartan-treated animals compared to controls, suggesting a shift in the local RAAS towards enhanced Ang II degradation, further contributing to the observed tissue-protective effects. Importantly, the absence of comparable effects in animals treated with finerenone further underscores that the deleterious effects observed are primarily driven by Ang II signaling rather than downstream mineralocorticoid receptor activation.

So, these findings offer novel insights into the pathophysiology of diabetic enteropathy and propose a mechanistic link between Ang II signaling and GI tissue alterations in diabetes. Gastrointestinal remodeling is a well-documented complication of diabetes, involving structural changes such as muscular hypertrophy, fibrosis, and altered neuromuscular function[50,87] as stated before. Ang II in known to exert multifaceted biological actions via activation of the AT_1R, including vasoconstriction, cell proliferation, inflammation, and fibrosis[111,112]. Within the GI tract, these effects translate into pathological changes in smooth muscle layers, ECM composition, and microvascular perfusion, all of which can contribute to GI dysmotility and remodeling, observed in diabetes[19,87]. In our study, losartan effectively prevented muscular remodeling in diabetic animals, suggesting a direct role of Ang II in the smooth muscle hypertrophy and ECM expansion. The profibrotic role of Ang II is largely known and is mediated through the induction of TGF-β1 and ROS-mediated pathways[111,112]. In our experimental model, the reduction in GI wall thickening with losartan likely reflects a combination of diminished reduced oxidative stress and probably TGF-β1 signaling, both downstream of AT_1R antagonism. Our results support this mechanistic model, as losartan-treated animals demonstrated improved redox status, reflected by normalization of tGSH and restoration of the GSH/GSSG ratio toward control values observed in control animals. These findings are in line with previous studies showing that Ang II contributes to oxidative stress through the stimulation of NADPH oxidase and subsequent ROS generation[110,125]. Given that oxidative stress is a well-established contributor to diabetic complications[110] and GI dysfunction[117,126], these results highlight another key mechanism through which losartan may exert its tissue-protective effects. ROS not only directly damage cellular components but also function as secondary messengers, amplifying inflammatory and fibrotic signaling pathways. Indeed, ROS-driven activation of NF-κB and AP-1 transcription factors enhances the expression of proinflammatory cytokines and adhesion molecules, fostering a local inflammatory environment[127]. The oxidative stress generated via Ang II contributes to mitochondrial dysfunction and impairs antioxidant defenses, particularly through depletion of GSH and reduced NADPH availability[110,125]. Our findings are consistent with earlier

studies in other organs showing that ARBs can preserve glutathione homeostasis and suppress NADPH oxidase and thus decrease organ fibrosis[128].

Another important finding in this thesis is the increased local ACE2 activity observed in the jejunum and ileum following losartan treatment. ACE2 catalyzes the conversion of Ang II into Ang 1–7, which exerts vasodilatory, antifibrotic, and antioxidant effects via Mas receptor[129]. This shift in the balance of local RAAS components indicates not only suppression of the harmful Ang II/AT_1R axis but also enhancement of the counter-regulatory ACE2/Ang-1–7/Mas pathway. The upregulation of ACE2 may occur as a compensatory mechanism in response to AT_1R blockade, but further studies are needed to elucidate the exact mechanism.

In contrast, animals treated with finerenone did not exhibit any protective effects on GI structure or redox balance. Despite its recognized anti-inflammatory and antifibrotic properties in other diabetic complications, such as diabetic nephropathy[130], finerenone failed to prevent GI remodeling or normalize GSH-related oxidative stress parameters. In line with this, previous studies have shown that aldosterone-induced effects on fibrosis are often dependent on prior Ang II signaling[131]. The inability of finerenone to replicate losartan's benefits underscores the centrality of Ang II in gastrointestinal pathophysiology in diabetes and supports the notion that mineralocorticoid receptor activation plays a less prominent role in the gastrointestinal manifestations of diabetes.

Importantly, our findings contribute to a growing recognition of the gastrointestinal tract as an active target of diabetic complications, where local RAS regulation may play a more significant role than previously appreciated. Although systemic RAAS alterations are well-characterized in diabetes, local tissue-specific RAAS systems - including that of the gut - can function independently and exert profound autocrine and paracrine effects[90]. The concept of tissue-specific RAAS activation explains why conventional systemic markers may fail to predict localized damage and reinforces the need to study local RAAS components when investigating organ-specific complications.

From a translational standpoint, the present data suggest that ARBs such as losartan could offer benefits in preventing diabetic gastrointestinal complications, potentially improving patient outcomes and quality of life. While gastrointestinal manifestations of diabetes, such as dysmotility, constipation, diarrhea, and bloating, are frequently underdiagnosed and undertreated[132], our findings highlight a mechanistically plausible and pharmacologically feasible approach to their prevention. Further studies in clinical settings are warranted to explore this therapeutic potential, as well as to delineate the long-term effects of RAAS modulation on gut function and structure in diabetic patients.

Concluding remarks and future perspectives

The work presented in this thesis has significantly advanced the understanding of gastrointestinal complications associated with diabetes, both in animal models and in companion animals (cats and dogs). Overall, we demonstrated that the gastrointestinal tract undergoes structural and functional remodeling in the context of diabetes. Importantly, this work provided novel insights into the local RAAS in the diabetic gut - a topic previously unexplored. Our findings indicate that the local RAAS behaves differently from the systemic RAAS: while both ACE and ACE2 are upregulated systemically, only ACE expression is increased locally. This suggests a shift towards enhanced local formation of Ang II, with potential pathophysiological consequences. Additionally, we observed clear indications of localized oxidative stress in the gastrointestinal tissue in models of both type 1 and type 2 diabetes, another novel finding in the field.

In pets, particularly in cats, we identified signs of gastrointestinal dysfunction and remodeling akin to those seen in humans. These alterations are likely to contribute to decreased animal welfare and may present additional challenges for diabetes management by pet owners.

Crucially, this study also demonstrated that pharmacological modulation of the RAAS can serve as a viable therapeutic strategy to mitigate these gastrointestinal alterations. This finding could be transformative, as current treatments for diabetic gastrointestinal complications are primarily symptomatic. In contrast, RAAS-targeted therapy has the potential to address underlying mechanisms and offers a more effective and disease-modifying approach.

Future perspectives for this research involve an even more comprehensive assessment of the local RAAS in diabetic gut. We have already collected portions of the entire gastrointestinal tract from diabetic STZ-induced animals treated with losartan and finerenone, which are now prepared for Ang II quantification. Given that TGF-β1 is a key regulator of fibrosis associated with Ang II, we also collected samples to quantify its expression via PCR and immunohistochemical evaluation.

Additionally, we have planned further experimental protocols in which animals will be maintained in a diabetic state for two weeks. This period has already shown to be sufficient for inducing significant gastrointestinal alterations. Following this, we will treat the animals with ARBs to investigate their potential therapeutic effects beyond prevention. We aim to explore various dosages to determine the most effective intervention. Despite not observing significant changes in the mucosal layers in animals treated with losartan, we also plan to expand our study to investigate the mucosa from alternative perspectives, such as absorption.

Furthermore, we intend to extend our study to explore gastrointestinal alterations in diabetic pets, with a focus on the impact of these alterations on both the animals and their owners. We are considering the use of a broader questionnaire-based approach to better understand how these changes influence the interaction between diabetic animals and their owners. In addition to continuing with ultrasound and histopathological evaluations, we aim to increase our sample size, particularly in dogs to strengthen our findings. Once we establish that diabetic pets exhibit gastrointestinal alterations, we will explore the effects of ARBs in a long-term therapeutic context.

The work developed during this thesis is currently in the process of securing an international patent and has garnered significant interest from a pharmaceutical company. We are in active discussions about the next steps to advance this research, with the goal of translating this knowledge into a solution that could potentially benefit both people and companion animals who suffer daily from the often-overlooked GI complications of diabetes. This work, grounded in the essential contributions of laboratory animals, has the potential to change the way we address diabetes-related gastrointestinal complications, transforming the landscape of treatment.

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Chapter 5 – Annexes

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Annex A: supplementary material from publication 2.3.

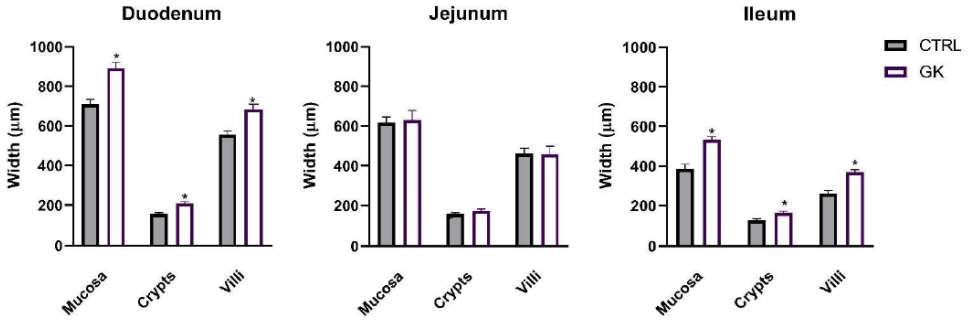


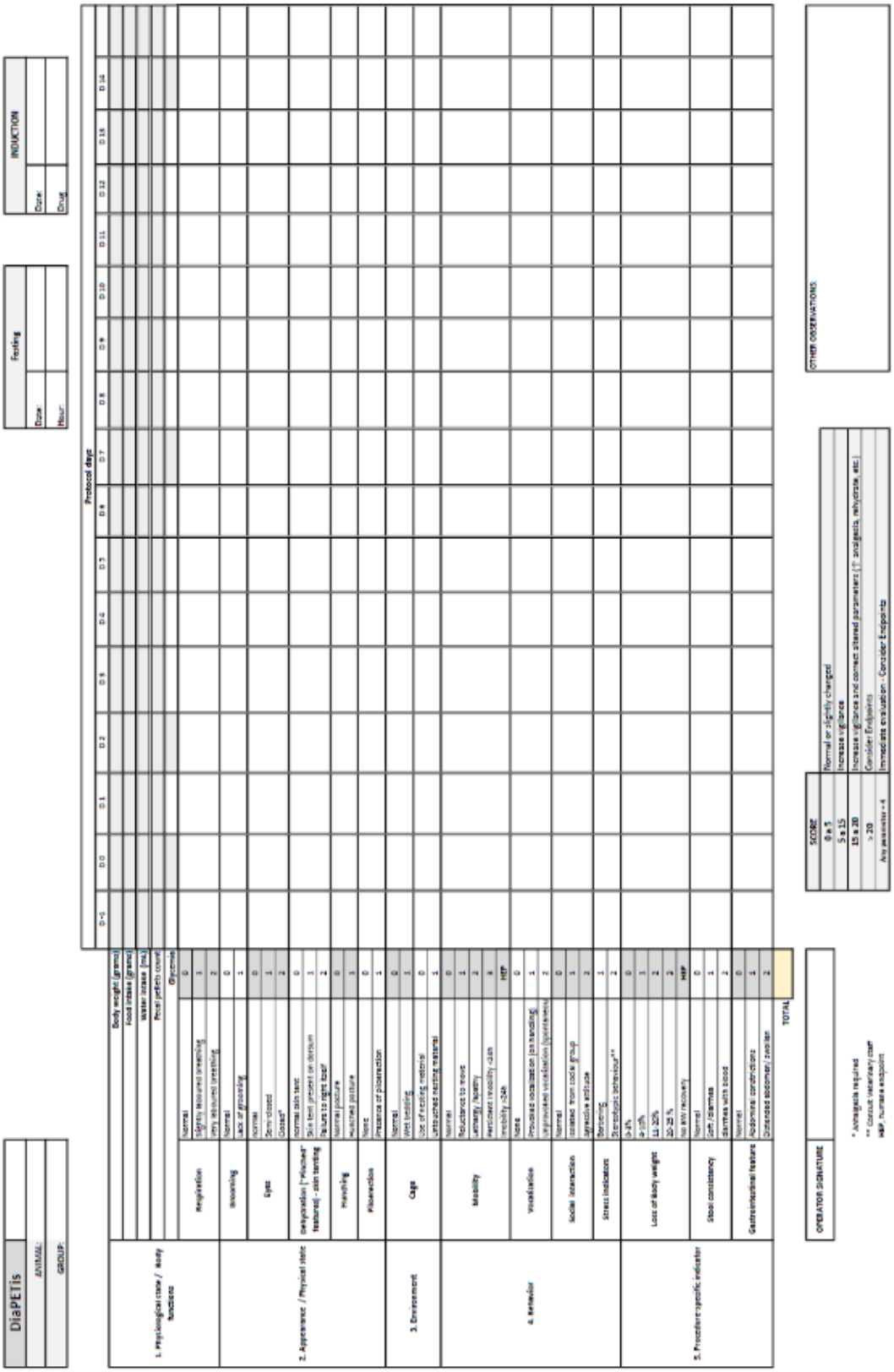
Figure S1. Morphometric analysis of mucosa, differentiated by crypt depth and villus height, of all intestinal segments (duodenum, jejunum, ileum) in control rats (CTRL, $n = 5$) and GK diabetic rats (GK, $n = 6$). Results are expressed as mean ± SEM. Comparisons between CTRL and GK groups were made using 2-way ANOVA followed by unpaired t-test with Welch's correction. *$p < 0.05$ indicates significant difference from corresponding control.

Annex B: supplementary material from publication 2.4.

S1: Video of voluntary oral administration of losartan

S2: Video of voluntary oral administration of finerenone

S3: Supervision table



Annex C: supplementary material from publication 3.2.

Questionário

Este questionário enquadra-se num projeto de investigação desenvolvido no âmbito do programa de doutoramento em Ciências Veterinárias do Instituto de Ciência Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP), intitulado "ESTUDO DA DISMOTILIDADE EM CÃES E GATOS DIABÉTICOS". Este estudo pretende compreender se cães e gatos diabéticos apresentam alterações digestivas, como as verificadas em outras espécies. Caso aceite participar, solicitamos o preenchimento deste questionário anónimo (máximo de 8 minutos).

Agradecemos desde já a sua colaboração.

1. Dados do seu animal de companhia

1.1. Espécie (assinale com uma cruz): Gato_____ Cão _____

1.2. Sexo (assinale com uma cruz): Fêmea_____ Macho _____

1.3. Idade: _____

1.4. Raça: _____

1.5. Peso (aproximado) em Kg: _____

1.6. O seu animal está castrado (assinale com uma cruz)?

Sim ____. Há quantos anos? _____

Não ____.

1.7. Há quantos anos o seu animal foi diagnosticado com diabetes?

_____

1.8. Historial médico e outras patologias associadas:

_____

1.9. Medicação atual (nome do medicamento, dose e número de vezes que o administra por dia):

_____

2. Sintomatologia associada à Diabetes

Desde que foi diagnosticado com Diabetes, o seu animal (assinale com uma cruz):

2.1. Aumentou o consumo de água? SIM ____ NÃO ____ NÃO SEI ____

2.2. Urina mais vezes ou em maior quantidade? SIM ____ NÃO ____ NÃO SEI ____

2.3. Come mais? SIM ____ NÃO ____ NÃO SEI ____

2.4. Perdeu peso? SIM ____ NÃO ____ NÃO SEI ____

3. Alterações Digestivas

Desde que foi diagnosticado com Diabetes, o seu animal (assinale com uma cruz):

3.1. Vomita mais vezes? SIM ____ NÃO ____ NÃO SEI ____

Caso não seja comum vomitar, salte para a questão 3.2

3.1.1. Quantas vezes vomita por semana?

_____

3.1.2. O vómito acontece quanto tempo após a refeição? _____

3.1.3. Após o vómito, o animal continua a querer comer? SIM ____ NÃO ____

3.2. Quantas vezes o seu animal defecava por dia antes de ser diagnosticado com diabetes?

_____

3.3. Verificou alguma alteração na frequência de defecação? SIM ____ NÃO ____

3.4. Se sim, aumentou ou diminui o número de vezes que defeca por dia?_____

3.5. Verificou alguma alteração no volume das fezes? SIM ____ NÃO ____

3.6. Se sim, o volume por defecação aumentou ou diminui?

_____

3.7. Houve algum episódio de diarreia? SIM ____ NÃO ____

3.8. Existe alguma alteração nos hábitos de defecação do animal (por exemplo, começar a defecar em casa quando antes não o faria, alteração da posição)? _____

_____

3.9. Existem sinais de tenesmo (vontade de defecar, percetível quando o animal se colocada na posição habitual, sem o conseguir fazer)? SIM ____ NÃO ____

3.10. Encontra alterações na aparência /cor /tamanho das fezes? SIM ____ NÃO ____
Quais? _____

3.11. Há alguma alteração digestiva que se tenha apercebido e que não tenha sido questionada? _____
_____
_____