PAPEL DEL MICROAMBIENTE INFLAMATORIO EN LA PROGRESIÓN DEL CÁNCER COLORECTAL Y LA DIABETES TIPO 2

ROLE OF THE INFLAMMATORY MICROENVIRONMENT IN THE PROGRESSION OF COLORECTAL CANCER AND TYPE 2 DIABETES

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Abstract

The microenvironment is becoming critical to understand how different cells behave in different contexts. The present thesis manuscript includes two chapters in which the phenotype of different immune cells is altered by changes in the microenvironment. The first chapter discusses the impact of type 2 diabetes (T2D) on adipose tissue mast cells while the second chapter focuses on the population dynamics and phenotypical changes of neutrophils, eosinophils, plasmacytoid dendritic cells, conventional dendritic cells, and mast cells in colorectal cancer.

Type 2 diabetes (T2D) is a rapidly growing global health concern, driven by factors such as poor dietary choices and sedentary lifestyles. It is associated with severe chronic conditions and significant strain on healthcare systems.

While adipocytes and macrophages were traditionally considered the primary contributors to adipose tissue expansion, recent research highlights the important role of mast cells in this process. Angiogenesis, critical for a physiological adipose tissue expansion, is compromised in T2D, contributing to hypoxic stress and inflammation. Mast cells, which release various pro-angiogenic factors, play a pivotal role in angiogenesis and glucose-dependent adipogenesis, contributing to metabolically healthy adipose tissue expansion. Nevertheless, there was some controversy since some authors claim that mast cells exacerbate the inflammatory response in adipose tissue during T2D while others claim that mast cells play a beneficial role.

The first chapter of this thesis manuscript shows that T2D reduce the number of mast cells in adipose tissue and the surface abundance of some important proteins for their physiological functioning: CD45, CD117, FceRI, and CD203c. Importantly, those changes were more prominent in the omental (o-WAT) than in the subcutaneous white adipose tissue (s-WAT). Therefore, contrary to previous notions, mast cells do not appear to be central drivers of adipose tissue inflammation in T2D.

Colorectal cancer (CRC) ranks as the fourth most prevalent global cancer and the second leading cause of cancer-related fatalities. The TME plays a pivotal role in CRC, encompassing a complex interplay of immune cells, stromal components, and signaling molecules that are essential for tumor origin and progression. Chronic inflammation within the TME, particularly in conditions like inflammatory bowel disease, contributes to CRC development. The second chapter of this thesis shows that neutrophils, plasmacytoid dendritic cells, conventional dendritic cells, and mast cells counts increase in the CRC compared to the normal mucosa, while the count of eosinophils decrease. Besides, neutrophils, eosinophils, plasmacytoid dendritic cells, and conventional dendritic cells decrease the surface abundance of CD45 in CRC. CD45 plays an important role in signal transduction in innate immune cells. Therefore, this may be novel mechanism of immune scape employed by the tumor.

Resumen

El estudio del microentorno celular se está volviendo fundamental para comprender cómo distintas células se comportan en diferentes contextos. Este manuscrito de tesis incluye dos capítulos en los que el fenotipo de diferentes células del sistema inmunológico se ve alterado por cambios en el microentorno. El primer capítulo discute el impacto de la diabetes tipo 2 (T2D) en los mastocitos del tejido adiposo, mientras que el segundo capítulo se centra en las dinámicas de poblaciones y los cambios fenotípicos de los neutrófilos, eosinófilos, células dendríticas plasmacitoides, células dendríticas convencionales y mastocitos en el cáncer colorectal (CRC).

La diabetes tipo 2 (T2D) es un problema de salud global que crece rápidamente impulsada por factores como dietas con excesivo aporte calórico y estilos de vida sedentarios. Está asociada con enfermedades crónicas graves y supone una carga significativa para los sistemas sanitarios.

Aunque tradicionalmente se consideraba que los adipocitos y los macrófagos eran las principales células implicadas en la expansión del tejido adiposo, investigaciones recientes resaltan el papel importante de los mastocitos en este proceso. La angiogénesis, crucial para la expansión fisiológica del tejido adiposo, se ve comprometida en la T2D, lo que contribuye a generar un estrés hipóxico e inflamación. Los mastocitos, que liberan diversos factores proangiogénicos, desempeñan un papel fundamental en la angiogénesis y la adipogénesis dependiente de la glucosa, contribuyendo a una expansión del tejido adiposo metabólicamente saludable. Sin embargo, ha habido controversia con respecto al papel de los mastocitos en el tejido adiposo en pacientes con T2D. Algunos autores sostienen que los mastocitos exacerban la respuesta inflamatoria en el tejido adiposo durante la T2D, mientras que otros afirman que desempeñan un papel beneficioso.

El primer capítulo de este manuscrito de tesis muestra que la T2D reduce el número de mastocitos en el tejido adiposo y la cantidad de algunas proteínas importantes para su funcionamiento fisiológico en su membrana plasmática: CD45, CD117, FceRI y CD203c. Es importante destacar que estos cambios son más prominentes en el tejido adiposo omental (o-WAT) que en el tejido adiposo subcutáneo blanco (s-WAT). Por lo tanto, a diferencia de las concepciones anteriores, los mastocitos no parecen tener un papel relevante en la inflamación del tejido adiposo en la T2D.

El cáncer colorrectal (CRC) se sitúa como el cuarto cáncer más prevalente a nivel mundial y la segunda causa principal de muertes relacionadas con el cáncer. El microentorno tumoral (TME) desempeña un papel fundamental en el CRC, abarcando una compleja interacción de células del sistema inmunológico, componentes estromales y moléculas señalizadoras que son esenciales para el origen y la progresión del tumor. La inflamación crónica en el TME, especialmente en condiciones como la enfermedad inflamatoria intestinal, contribuye al desarrollo del CRC. El segundo capítulo de este manuscrito de tesis muestra que la cantidad de neutrófilos, células dendríticas plasmacitoides, células dendríticas convencionales y mastocitos aumentan en el CRC

en comparación con la mucosa normal, mientras que el recuento de eosinófilos disminuye. Además, los neutrófilos, eosinófilos, células dendríticas plasmacitoides y células dendríticas convencionales reducen la abundancia superficial de CD45 en el CRC. CD45 desempeña un papel importante en la transducción de señales en las células del sistema inmunológico innato. Por lo tanto, esto podría ser un mecanismo novedoso de evasión inmunológica empleado por el tumor.

Overview

Homeostasis is the ability of the body to maintain a stable internal environment despite external changes and disruptions. This state of dynamic stability is crucial for the proper functioning of various physiological processes in the body (1). The disruption of homeostasis triggers inflammation, which is a protective response mounted by the immune system that aims to restore homeostasis (2). The primary objective of an inflammatory response is to eliminate the harmful stimuli that have triggered the inflammation in the first place and initiate a repair process in the affected tissue. When this is accomplished, the tissue returns to its previous homeostatic state and inflammation ceases. However, it may happen that the immune system is not able to eliminate the cause of inflammation effectively (2). When this occurs, inflammation displaces the tissue to a new homeostatic state, in which the tissue reaches a new equilibrium (3). This homeostatic shift can be done in two ways: isolating the cause of the inflammation or promoting a metabolic adaptation of the tissue.

Firstly, sometimes it is possible to physically isolate the cause of inflammation, creating a barrier that prevents further damage to the tissue (4). A classic example of this is the formation of granulomas in the lungs of patients with tuberculosis. When the immune system is not able to effectively remove Mycobacterium tuberculosis, macrophages in the affected area fuse to form a barrier around bacteria, which is supported by an external layer of T lymphocytes, B lymphocytes, neutrophils, eosinophils, mast cells, dendritic cells, and fibroblasts (5). This structure, called granuloma, prevents the spreading of the bacteria. In this context, the tissue cannot go back to the previous homeostatic state because part of the previous healthy tissue has been replaced by the granuloma and also, it has to devote resources to maintain the barrier and sustain the ongoing immune response. Nevertheless, the tissue reaches a new homeostatic state that allows it to keep functioning.

Secondly, sometimes the cause of the inflammation cannot be physically isolated, but it is possible to induce a metabolic adaptation of the tissue to cope with this challenge (3). A typical example of this is exercise-induced inflammation. During intense physical activity like weightlifting, muscles undergo micro-injuries that trigger an inflammatory response. This inflammation is necessary to heal the damaged muscle fibers (6). If the person does the weightlifting just one time without repeating the exercise, the inflammation will resolve, and the tissue would return to its previous homeostatic state (7). However, if the person does weightlift repeatedly over time, the inflammatory response does not have enough time to restore the previous homeostatic state, which generates more stress on the tissue (7). Instead, inflammation promotes the metabolic adaptation of the muscle fibers, which leads to fiber hypertrophy and increased physical capacity (6,7). Therefore, in this case, inflammation shifts the tissue from its previous homeostatic state to a new and improved state that allows for increased strength and performance.

Importantly, inflammation is not a homogeneous phenomenon but rather encompasses a wide range of processes with diverse consequences. In general, there are three types of inflammatory processes with different triggers, physiological purposes, and pathological consequences when dysregulated (8). The first type of inflammation, and the most studied, is the inflammation developed to remove an insult, like an infection. The physiological purpose of this inflammation is to defend the host against pathogens like viruses and bacteria. The pathological consequences of the dysregulation of this type of inflammation include autoimmunity and inflammatory tissue damage (8). This type of inflammation has been widely studied and it is out of the scope of this thesis to delve into the intricate details of its mechanisms and consequences.

The second type of inflammation is triggered by tissue injury and its purpose is to activate the tissue repair response. The pathological consequences of its dysregulation include fibrosis, metaplasia, and tumor progression (3). An example of this could be found in the colon. The colon is lined with a layer of cells called the epithelium, which protects the underlying tissue from harmful substances (9). When this epithelial layer is injured, an inflammatory response is triggered to facilitate tissue repair (9). In this inflammatory response, resident innate immune cells recruit different subpopulations of peripheral T lymphocytes, which produce IL-22 to promote the proliferation and differentiation of epithelial cells to restore the integrity of the epithelial layer (10,11). This process is enhanced by Th2 lymphocytes through the release of IL-4, IL-5, and IL-13 which promote tissue remodeling (12). Besides, Th2 lymphocytes and M2 macrophages prevent the activation of the type of inflammation described in the previous paragraph through the release of cytokines like IL-10 and TGF-β (12–14). Finally, mast cells and fibroblasts promote extracellular matrix remodeling and tissue regeneration through the secretion of growth factors (15). However, colorectal cancer (CRC) can take advantage of this type of inflammation and keep it permanently activated to progress. It can use IL-22 to increase proliferation (16,17), Th2 and M2 activation to suppress the antitumoral immune response (13,14), and mast cells and fibroblast cells to promote angiogenesis and tissue remodeling (18,19).

Finally, the third type of inflammation is triggered by tissular stress and malfunction. In this type of inflammation, the physiological purpose is to adapt the tissue to the stress (8). Noteworthy, this type of inflammation is typically low-grade and persistent, and it has been proposed to be referred to as para-inflammation (20). Due to the low intensity of this type of inflammation, it is mainly controlled by resident cells like macrophages and mast cells, rather than recruiting immune cells from the bloodstream (3). The pathological consequences of the dysregulation of this type of inflammation include the deleterious shift in homeostatic set points, and the development of chronic diseases due to this new homeostatic set point (8). Here, the typical example would be insulin resistance in adipose tissue and type 2 diabetes (T2D). Under homeostatic conditions, when a person has sporadically an elevated blood glucose level, the pancreatic beta cells respond by releasing insulin to bring the glucose levels back to normal (21). This generates mild stress that triggers inflammation in adipose tissue so that adipocytes can expand to store the excess energy and maintain glucose homeostasis (22). This inflammation is needed for the proper functioning of the tissue in response to stress and ceases when all this excess energy is stored. Thanks to this inflammation lipids are redistributed between adipocytes

with the help of foam cells (a special subtype of macrophages), progenitor cells differentiate into adipocytes, and, if needed, new capillaries are formed to support the increased metabolic demand (23). Importantly, murine models have shown that in the absence of this inflammation, adipose tissue is unable to properly store the excess of energy and causes ectopic lipid accumulation, glucose intolerance and systemic inflammation (24).

However, when this elevated glucose stress persists over time, the excess of caloric intake and adipocyte expansion lead to tissular stress and malfunction, triggering para-inflammation in adipose tissue (25). This para-inflammation alters the adipokine secretion profile, reducing anti-inflammatory adipokines and increasing the production of pro-inflammatory adipokines (25). Such impairs the endocrine function of adipose tissue and promotes the moderate recruitment of peripheral immune cells. This recruitment of immune cells further exacerbates the inflammatory response and creates a positive feedback loop, leading to chronic inflammation in adipose tissue (22,23). This causes a shift from the homeostatic state (insulin sensitivity) to a new state (insulin resistance). This new state allows adipose tissue to adapt to the new environment by deviating the excess of energy (glucose and lipids) that it cannot handle to other tissues, such as the liver and skeletal muscles (26). This shift in energy distribution and the development of insulin resistance has significant chronic pathological consequences, including increased risk of cardiovascular diseases, metabolic syndrome, and obesity-related complications (27).

In short, there are different triggers and mechanisms underlying various types of inflammation. The knowledge of these inflammatory processes has allowed us to develop new approaches to modulate specific types of inflammation, including neutralizing antibodies, recombinant cytokines, small-molecule inhibitors, DC vaccines, oncolytic viruses, and TLR agonists (28). However, there is still much more to learn about how these inflammatory processes are regulated and their intricate relationship with physiological and pathological consequences. A better understanding of these processes will enable the development of more targeted and effective therapeutic interventions for inflammatory conditions and associated diseases.

Chapter 1: Type 2 diabetes alter the number and phenotype of mast cells in white adipose tissue

Introduction

Type 2 diabetes (T2D) constitutes 90% of all diabetes cases, with its incidence quadrupling over the past three decades (29). It stands as the ninth leading global cause of death and is projected to affect 693 million individuals by 2045 (30). T2D is characterized by insulin resistance and impaired glucose metabolism, leading to chronically elevated blood sugar levels (hyperglycemia) (31). The surge in T2D prevalence is primarily attributed to poor dietary choices and sedentary lifestyles (29). Notably, uncontrolled T2D can trigger severe chronic conditions, including cardiovascular disease, kidney disease, and neuropathy (32,33), significantly straining healthcare systems.

Insulin is produced by beta cells in the pancreas in response to elevated blood glucose levels, and it promotes the uptake and utilization of glucose by cells throughout the body. Insulin acts mainly in metabolic tissues (such as muscle, liver, and adipose tissue) (34). When it binds its receptor on target cells, it initiates a cascade of intracellular signals that ultimately result in GLUT4 translocation to the cell membrane, allowing glucose to enter the cell (35). Insulin resistance occurs when cells of metabolic tissues translocate fewer GLUT4 transporters to the plasma membrane than normally in response to insulin, leading to decreased glucose uptake and elevated blood glucose levels (34,35).

Insulin resistance is evolutionary well-preserved (36,37) as it serves as a mechanism to ensure that glucose is preserved for vital organs (like the brain) during times of stress or famine (38). During the Ice Age, the human diet included high amounts of proteins and low amounts of carbohydrates, which made insulin resistance a beneficial feature for survival (39). With agriculture, the amount of dietary carbohydrates increased. However, most of these carbohydrates were complex and required enzymatic breakdown for absorption, limiting their immediate availability as glucose. Therefore, the increase in plasma glucose levels after a meal was slower, allowing insulin enough time to mediate its effects on glucose uptake by cells (39). After the Industrial Revolution, processed foods with high levels of refined carbohydrates became widely available, resulting in increased consumption of easily digestible glucose (39). In this context, insulin resistance became a disadvantageous feature, as the constant high intake of glucose led to persistent stimulation of insulin production and subsequent desensitization of insulin receptors on target cells (34,35).

As discussed until now, the direct physiological consequence of insulin resistance is hyperglycemia. In the initial stages of T2D, the reduced efficacy of insulin is compensated by an

increase in pancreatic beta-cell function and secretion of insulin, leading to hyperinsulinemia (31). This compensatory response aims to maintain normal blood glucose levels. As the disease progresses beta cells become exhausted, as they are unable to sustain the increased demand for insulin production, leading to a decline in insulin secretion and finally to beta cell dysfunction (31). Chronic hyperglycemia affects blood vessels generating advanced glycation end products, which promote oxidative stress and inflammation (40). This can damage small blood vessels like the ones located in the eyes, kidneys, and nerves, leading to microvascular complications such as diabetic retinopathy, nephropathy, and neuropathy (Beckman, 2016). When chronic hyperglycemia persists, it also affects larger blood vessels and can result in macrovascular complications such as cardiovascular disease, stroke, and peripheral artery disease (41).

Type 2 diabetes and adipose tissue

Adipose tissue can be subclassified into brown adipose tissue (BAT) and white adipose tissue (WAT). BAT is responsible for energy expenditure and heat production through the process of thermogenesis (42). In this process, brown adipocytes generate heat by uncoupling oxidative phosphorylation from ATP synthesis in the mitochondria, through the action of uncoupling protein 1 (43). Alternatively, WAT is in charge of energy storage (42). In humans, BAT is primarily found in newborns (in the cervical-supraclavicular region, periaortic areas inside the thorax and the abdomen, and in the perirenal fat) (44), and it gradually decreases with age and is mostly replaced with WAT (44,45). In adults, some WAT adipocytes can undergo a process known as browning or beiging, in which they acquire a phenotype similar to brown adipocytes, including the ability to generate heat through thermogenesis (46). This phenotypic change occurs after the stimulation of the β3 adrenergic receptor in adipocytes, and it occurs in response to cold exposure (47).

WAT, the reservoir for excess caloric intake, primarily stores energy in the form of neutral lipids. Prolonged positive caloric imbalances require the expansion of adipose tissue to store that excess of energy (48,49). This can occur in different WAT depots depending on. Adipose tissue expansion is a complex process influenced by adipocyte precursor cells, hormones, adipokines, adipose tissue blood flow, and age. The number and potential of adipocyte precursor cells in WAT determines the capacity to generate new adipocytes **(Zubiría, 2017)**. In addition to insulin other hormones also contribute to adipose tissue expansion. Their role highly depends on the microenvironmental context, but in general thyroid hormones and catecholamines inhibit WAT expansion, while estrogen promotes it, and glucocorticoids regulate both adipogenesis and lipolysis depending on the molecular context (50–52). Adipokines are bioactive molecules secreted by WAT that play a crucial role in regulating metabolism and inflammation locally and systemically (52). Adipose tissue blood flow is critical to deliver the nutrients and oxygen required for WAT expansion and to remove waste products generated during the process (53). Finally, age can also impact adipose tissue expansion, as the capacity for adipocyte hyperplasia decreases with age (54).

WAT is usually subclassified depending on its anatomical location. These include subcutaneous white adipose tissue (s-WAT), which is located just beneath the skin, and visceral white adipose tissue (v-WAT), which surrounds internal organs (55). In the abdominal cavity, v-WAT is further subclassified into two main depots: omental white adipose tissue (o-WAT) and mesenteric white adipose tissue (m-WAT) (56). O-WAT is a sheath of WAT that hangs from the stomach and covers the intestines, while m-WAT is located in the mesentery, which supports the intestines and holds them in place (57). Despite their close proximity, o-WAT and m-WAT are very different. M-WAT is much more vascularized than o-WAT and is more metabolically active (58,59). However, o-WAT is much more expanded in obesity (59,60), suggesting a higher storage capacity for lipid accumulation. Besides, while o-WAT expansion is clearly associated with increased cardiometabolic risks, the role of m-WAT in these health outcomes is not yet fully understood (61). In humans, there is limited information on m-WAT since o-WAT is more associated with pathological conditions and also because it is more difficult to obtain samples of m-WAT due to its deep location within the abdominal cavity and higher risks associated with surgical procedures (62). Therefore, the present work will focus on s-WAT and o-WAT.

Different WAT depots exhibit distinctive expression profiles and secretory behaviors (49). S-WAT shows a higher expression of genes related to adipogenesis and lipid droplet formation than omental white adipose tissue (o-WAT). The consequence is that o-WAT has less potential to generate more adipocytes, store lipids, and breakdown lipids. Therefore, when o-WAT expands, the amount of free fatty acids in the tissue increases. Consequently, o-WAT accumulation results in higher free fatty acid levels than s-WAT, leading to lipotoxicity and inflammation (63,64). Hence, o-WAT expansion poses a risk factor for cardiometabolic diseases and T2D (65).

Traditionally, adipocytes and macrophages were viewed as the primary contributors to adipose tissue expansion. However, recent studies from various authors have emphasized the pivotal role of mast cells in this process. Adipocytes, responsible for energy storage, uptake carbohydrates and lipids, and store them as triacylglycerides (48). To accommodate the positive caloric imbalance and enhance adipocyte storage capacity, adipose tissue must expand. Such can be achieved through either adipocyte hypertrophy or hyperplasia (23,66). These pathways coexist, although one often predominates depending on the tissue microenvironment. Importantly, each of them leads to distinct metabolic outcomes.

Adipocyte hyperplasia increases the number of adipocytes, insulin sensitivity, and adiponectin secretion while reducing the production of pro-inflammatory cytokines. It also relies on efficient angiogenesis to prevent hypoxia. Conversely, adipocyte hypertrophy enlarges adipocyte size and pro-inflammatory cytokine secretion, reducing insulin sensitivity and adiponectin secretion. Importantly, adipocyte hypertrophy does not produce angiogenesis. Therefore, as hypertrophic adipocytes increase their size, they move away from blood vessels. If this is sustained over time, it leads to hypoxia-induced adipocyte necrosis, escalating tissue inflammation and free fatty acid release (23,66).

Resident macrophages in adipose tissue contribute to maintain adipose tissue homeostasis by clearing senescent adipocytes and lipoproteins. These macrophages, upon lipid ingestion,

differentiate into foam cells and help to regulate lipid exchange with adipocytes to prevent the accumulation of toxic free fatty acids in the extracellular space (67,68). Moreover, under normal conditions, foam cells adopt an M2 phenotype, promoting adipose tissue homeostasis. However, in cases of extensive adipocyte hypertrophy, insufficient vascularization leads to hypoxic adipocyte death via necrosis, triggering tissue inflammation. In this context, foam cells shift toward an M1 phenotype (68). Prolonged adipocyte hypertrophy exacerbates hypoxia, resulting in greater adipocyte necrosis and the release of pro-inflammatory cytokines and free fatty acids, further impairing tissue function (23,66–68). Besides, inflammation also promotes mitochondrial dysfunction (69) and adipokine dysregulation in adipocytes (70), enhancing tissue malfunctioning. In a nutshell, inflammation impacts WAT functioning and the response of WAT to that stress is more inflammation, generating a cycle of inflammation and dysfunction. In o-WAT, free fatty acids reaching the portal system and liver can induce hepatic toxicity, contributing to fatty liver disease and hepatic steatosis (64,71,72).

The role of mast cells in adipose tissue

As mentioned previously, the choice between adipocyte hyperplasia and hypertrophy depends mainly on the tissue angiogenic capacity (23,66). Noteworthy, angiogenesis is compromised in the adipose tissue of T2D patients (73). While angiogenesis is a multifaceted process, mast cells are crucial contributors, releasing proteases to create space for vessels, pro-angiogenic factors (like VEGF), FGF, TGF-beta, histamine (to enhance vascular permeability), and heparin (22).

Mast cells play a versatile role in their microenvironment, harboring a plethora of receptors to respond to changes in the microenvironment (74,75). These cells store granules with various cargo that denote their internal contents (74,76). Activation of different receptors on mast cells can prompt the release of distinct granules or vesicles, influencing the microenvironment either directly or through interactions with other cell types (74).

Mast cells, once primarily seen as inflammatory cells linked to allergies, are now recognized for their vital physiological roles, including the release of growth factors and other bioactive molecules in response to microenvironmental signals (74). Within adipose tissue, mast cells promote angiogenesis, lipid uptake by macrophages, and foam cell formation (22). Additionally, they respond to elevated glucose levels by secreting 15-deoxy-delta prostaglandin J2, which binds to PPARγ in pre-adipocytes, inducing their differentiation into adipocytes (77,78). In summary, mast cells in adipose tissue contribute to maintaining normoxia, clearing free fatty acids, and facilitating adipose tissue expansion through hyperplasia.

T2D induces changes in the adipose tissue microenvironment, including the recruitment of proinflammatory leukocytes from peripheral blood and alterations in the metabolomic profile (64,79). These changes affect resident cells, including mast cells. While some experiments in mice have yielded contradictory results (80,81), our understanding of mast cells in human T2D remains limited. Therefore, it is still unclear the dynamics of mas cells in T2D and and how T2D affect their phenotype.

Hypothesis

T2D negatively affect the population dynamics of mast cells in o-WAT and s-WAT and it will affect the surface abundance on CD45, CD117, FceRI, and CD203c too.

Objectives

- To count the amount of mast cells in o-WAT and s-WAT of patients with T2D, without TD2, and with an intermediate state.
- To measure the surface abundance of mast cells in o-WAT and s-WAT of patients with T2D, without TD2, and with an intermediate state.

Materials and Methods

Biochemical Parameters

For each patient, two blood tests were analyzed. In the first one, 6-9 months before the surgery, only fasting plasma glucose was analyzed. In the second one, just before the surgery, a complete analysis was performed. Blood tests were conducted in San Cecilio University Hospital by the clinical analysis laboratory within 24 hours. All tests were performed following approved protocols.

Cohort

For this study, 196 patients from 2 projects were recruited in the San Cecilio University Hospital. All patients had morbid obesity and underwent bariatric surgery (gastric bypass or gastric sleeve). Patients were stratified into three groups (non-T2D, pre-T2D, and T2D) following the criteria of the American Diabetes Association. Patients were excluded from the cohort if they present at least one of the following disorders: type I diabetes, drug-induced diabetes, genetic diabetes syndromes, diseases of the exocrine pancreas, and autoimmune diseases. All patients provided their written informed consent.

The first project included 100 patients and was focused on quantifying the number of mast cells in adipose tissue, while the second project included 96 patients and was focused on identifying alterations of the surface abundance of CD45, CD117, FceRI, and CD203c on mast cells in adipose tissue.

Sample Processing

Each patient gave two biopsies of adipose tissue from laparoscopic bariatric surgery at San Cecilio University Hospital (Granada, Spain). One biopsy was taken from the greater omentum, close to the stomach (o-WAT, omental white adipose tissue). The second biopsy was obtained near the surgical incision (s-WAT, subcutaneous white adipose tissue). After their extraction, both biopsies were conserved in ice and separated plastic jars with PBS. Later, visible blood vessels were eliminated from the samples. After, 2-2.5 g of each sample was weighed and cut into tiny portions. Subsequently, cut biopsies were digested in 10 ml of RPMI 1640 medium supplemented with 2 mg/ml type I collagenase (Sigma) and 5 mM $CaCl_2$, for 2h, at 37°C. Then, each sample was washed with 35ml of PBS and filtered through a 1 mm sieve. After, samples were centrifuged at 900 x g for 10 minutes and spilled through a 100 µm filter. Later, the samples were centrifuged again at 900 x g for 10 minutes, and the pellet was kept since it contains the stromal vascular fraction. Finally, the pellet was resuspended in 500 µl of antibody staining buffer (PBS, 2% fetal bovine serum, 0.09% albumin, and 0.05% sodium azide) and mixed with an internal standard. In the firs project, the internal standard employed was BD Truecount Absolute Counting Tubes while in the second project the internal standard used was CountBright Absolute Counting Beads. In both cases, the internal standards were handled following manufacturer instructions. We decided to change the internal standard since BD Truecount Absolute Counting Tubes showed heterogeneous emission intensity. This does not affect quantification since the internal control has size/complexity values far from any other cytometric population, it cannot be misled with cells, and thus fluorescent emission is not required for quantification. Regarding CountBright Absolute Counting Beads, it contains a suspension of autofluorescent beads with emission occurring through a wide spectrum of wavelengths with homogeneous intensity. This ensures a much more accurate quantification of the surface abundance of the different proteins.

Antibody Staining and Flow Cytometry

The resuspended stromal vascular fraction was stained with 2 µl of fluorophore-conjugated antibodies or controls in Eppendorf tubes for 20 minutes at room temperature. Later, samples were fixed, and erythrocytes were lysed adding 1 ml of BD FACS Lysing Solution for 30 minutes. After that, samples were centrifuged at 3500 x g for 10 minutes, and pellets were resuspended in 500 µl of PBS. Then, samples were stored until the next day at 4°C. A FACS ARIA III equipment was employed to perform the flow cytometry, and data were acquired on a logarithmic scale. The fluorescence of the internal standard was used to normalize the signal obtained from the fluorophore-conjugated antibodies. The antibodies employed are detailed in the annex. Mast Cells were identified as CD45+ CD117+ cells.

RNA Purification and RT-qPCR

Total RNA was purified for each adipose tissue biopsy using the RNeasy Mini Kit (Qiagen). Five hundred nanograms of RNA were retrotranscribed to cDNA using the iScript™ cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. The quantification of mRNA concentration for each gene was performed in a fraction of cDNA volume by Real-time PCR (CFX96 Real-Time System, BioRad) using the SsoFast EvaGreen Supermix (BioRad).

The primers (Supplementary Table 1) were tested previously to evaluate their specificity and sensitivity. Unspecific amplification was not detected in the test. To prevent amplification from eventual genomic DNA contamination, primers were designed to hybridize in different exons. Furthermore, when different transcript variants are described, primers were designed using the common sequence among them.

The annealing temperature was 65 °C. Each determination was carried out in duplicate, and the mathematical relation between the threshold cycle (Ct) level and the initial DNA quantity was evaluated by a standard curve. Finally, the results were normalized using the expression level of two housekeeping genes: PPIA (Peptidylprolyl isomerase A) and RPS13 (Ribosomal Protein S13).

Statistical Analysis

Kolmogorov-Smirnov test was used to test the normal distribution of data, and the Levene test was employed to check the homoscedasticity of the groups. To evaluate the differences between non-T2D, pre-T2D, and T2D groups, the one-way ANOVA test was used followed by a Tukey HSD. Student's t-test for paired samples was employed to analyze the differences between o-WAT and s-WAT, in non-T2D, pre-T2D, and T2D groups independently. Besides, we studied the internal structure of the data using the principal component analysis (PCA), multiple regression analysis, linear discriminant analysis, and random forest analysis. In the linear discriminant analysis and random forest analysis, we did not include the HbA1c variable because it is what defines the groups. P-values below 0.05 were considered significant. All tests were conducted with R software.

Results

Cohort baseline characteristics

Table 1 shows mean data for age, sex, hypertension, body mass index, waist-hip index, insulin, blood glucose, glycosylated hemoglobin (HbA1c), HOMA-IR, triglycerides, cholesterol, LDL, and HDL. In this study, women constituted 66% of the group, which might be related to social constraints in our geographic area. Notably, mean HbA1c was lower in the T2D group. The guidelines of the American Diabetes Association indicate that the goal for people with T2D should

be to have a HbA1c below 7%, as this can significantly reduce the risk of cardiovascular disease. Therefore, the patients with T2D in the present study had a mild condition (82).

Table 1. Baseline characteristics of the cohort. The values are expressed as mean ± standard deviation. T2D (type 2 diabetes) n=196.

	Non-T2D	**Pre-T2D**	**T2D**
Number of patients	75	64	57
Age (years)	43,2±9.9	47.6±12.4	51.3±10.2
Male/Female)	28/47	22/42	15/42
Hypertension (Yes/No)	40/35	42/22	42/15
Body Mass Index (kg/m^2)	45.1±7.1	45.2±7.5	43.5±6.6
Waist/Hip Index	0.89±0.09	0.93±0.10	0.94±0.12
Insulin (units/ml)	4.5±3.4	7.8±6.2	10.2±7.2
Glucose (mg/dl)	87.3±9.8	98.7±15.6	155.5±50.3
HbA1c (%)	5.3±0.5	5.8±0.5	6.7±1.1
HOMA-IR	1.04±0.76	1.96±1.70	3.89±3.13
Triglycerides (mg/dl)	138.2±55.8	162.1±73.2	149.7±39.3
Cholesterol (mg/dl)	162.2±41.1	152.1±29.7	148.8±48.3
LDL (mg/dl)	93.5±33.0	84.2±24.9	87.2±43.8

HDL (mg/dl)	41.0±11.4	36.3±10.2	37.2±12.4

White adipose tissue but not colonic mucosa have a population of mast cell progenitors

Mast cells throughout the cohort were identified by flow cytometry as $CD45^+$ $CD117^+$ $CD203c^+$ $Fc\epsilon RI^+$ (Figure 1A-D). In addition, the mast cell $CD34^+$ integrin $\beta7^+$ subset was analyzed in a subset of 15 patients. This subpopulation was found in both types of WAT in these 15 patients (Figure 1E). In contrast, this subpopulation was absent in five colonic mucosal samples (Figure 1F). Previous studies have shown that this subpopulation consists of mast cell progenitors in peripheral mouse tissues (83,84) and human blood (85). In addition, a heterogeneous pool of stem cells in WAT in humans has been reported to contain cells that can give rise to mast cells (86). Thus, the expression of CD34 and integrin $\beta7$ in a small subset of the mast cell pool strongly suggests the existence of a stable stem cell pool associated with the mast cell lineage in human WAT.

These mast cell progenitors were more abundant in o-WAT than in s-WAT and appeared to be reduced in both WAT types in the T2D group (Table 2). The proportion of mast cell stem cells in the total population is shown in Table 3.

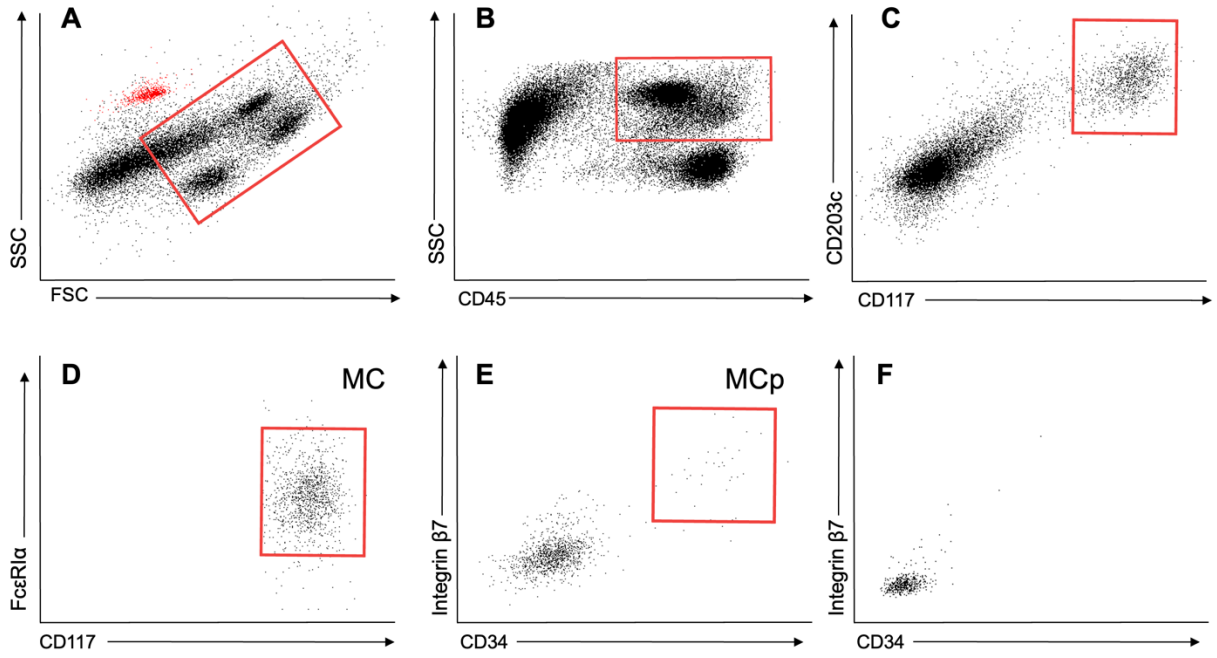

Figure 1. Flow cytometry. A-E) Gating strategy employed in the flow cytometry to distinguish mast cells and their progenitors in white adipose tissue. F) Gating of mast cell progenitors in colon mucosa. MCp (mast cell progenitors).

Table 2. Mast cell progenitors count/g of white adipose tissue. The values are expressed as mean ± standard deviation. T2D (type 2 diabetes), o-WAT (omental white adipose tissue), s-WAT (subcutaneous white adipose tissue) n=15.

	Non-T2D	**Pre-T2D**	**T2D**
o-WAT	1623.45 ± 3360.70	880.07 ± 1050.14	62.90 ± 24.55
s-WAT	596.23 ± 999.85	302.72 ± 145.38	60.2 ± 30.94

Table 3. Percentage of mast cell progenitors in the whole mast cell pool. The values are expressed as mean ± standard deviation. T2D (type 2 diabetes), o-WAT (omental white adipose tissue), s-WAT (subcutaneous white adipose tissue) n=15.

	Non-T2D	**Pre-T2D**	**T2D**
o-WAT	2.54% ± 4.29	3.04% ± 2.54	0.93% ± 0.75
s-WAT	2.47% ± 1.79	2.04% ± 1.56	0.57% ± 0.27

Reduced number of mast cells in omentum and subcutaneous white adipose tissue in patients with T2D

Figure 2Figure 3 show that the number of mast cells was lower in the T2D group in both o-WAT and s-WAT. These results suggest that both o-WAT and s-WAT mast cells are negatively affected by dysregulation of glucose metabolism, especially when reaching T2D. Nevertheless, T2D seems to have a bigger effect on o-WAT than in s-WAT.

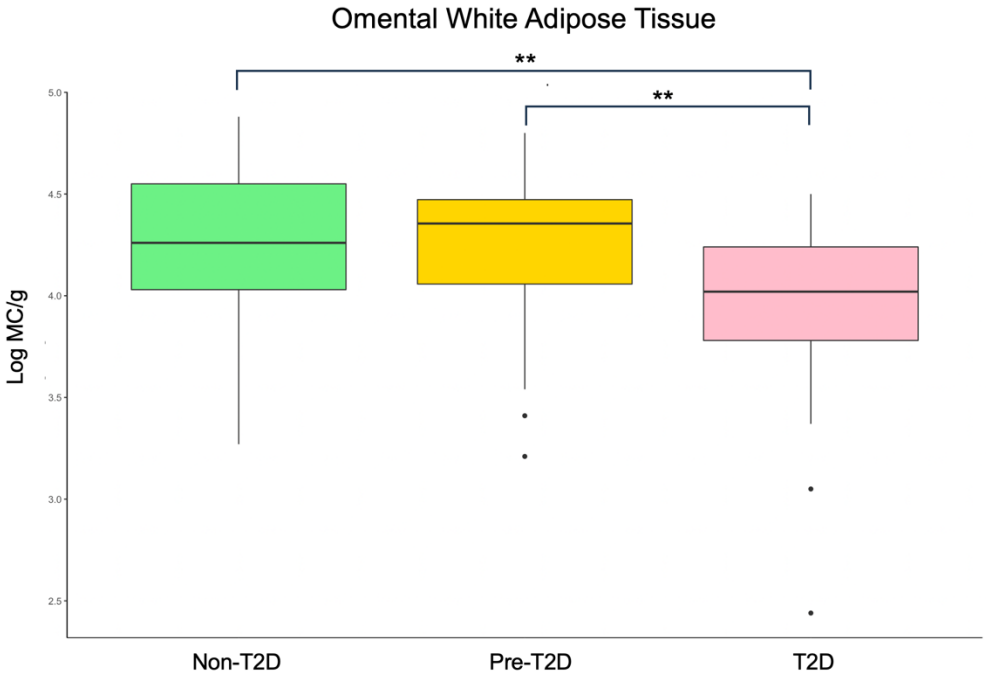

*Figure 2. Comparison of MC/g of omental white adipose tissue depending on T2D status. T2D (type 2 diabetes), MC (mast cells), "**" (p-value < 0.01), n=100.*

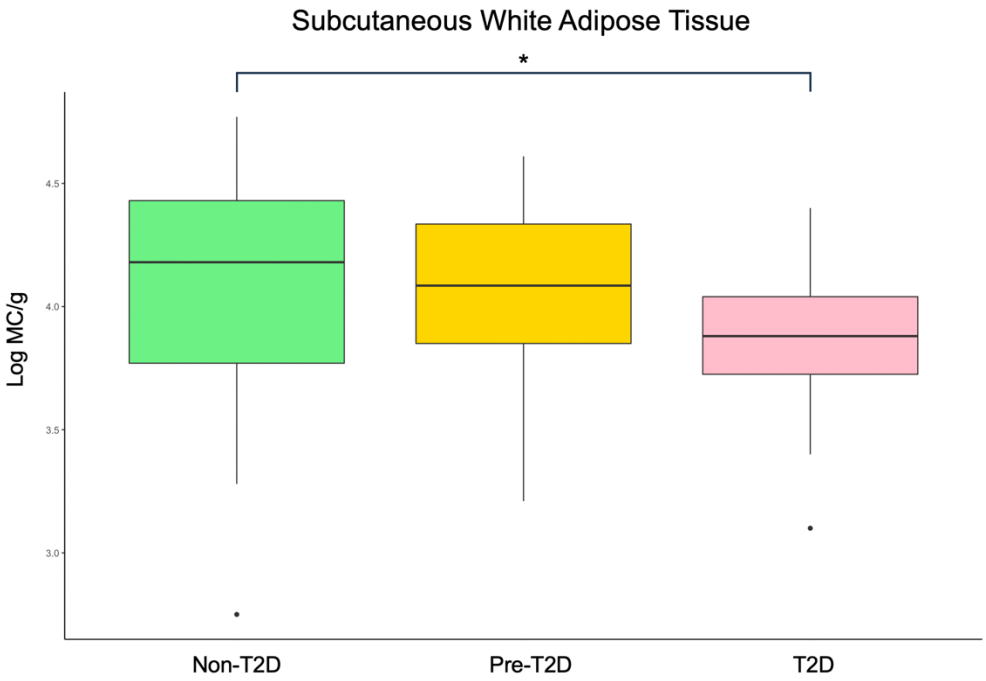

*Figure 3. Comparison of MC/g of subcutaneous white adipose tissue depending on T2D status. T2D (type 2 diabetes), MC (mast cells), * (p-value < 0.05), n=100.*

There are differences in the number of mast cells in the omental and subcutaneous white adipose tissue between the non-T2D and pre-T2D groups, but not in the T2D group

Figure 4Figure 5, Figure 6 indicate that the difference in the counts of mast cells between o-WAT and s-WAT is reduced in patients with T2D. Generally, the number of mast cells in o-WAT is greater than in s-WAT. This probably occurs because o-WAT, but not s-WAT, is exposed to microbial products of the gut microbiome (87–89). Besides, o-WAT also plays a critical role in peritoneal immune responses (87–89). Nevertheless, although the number of mast cells in the T2D group has decreased rapidly, they are not impacted in the same way. These results suggests that the effects of T2D on mast cell counts are higher in o-WAT than in s-WAT.

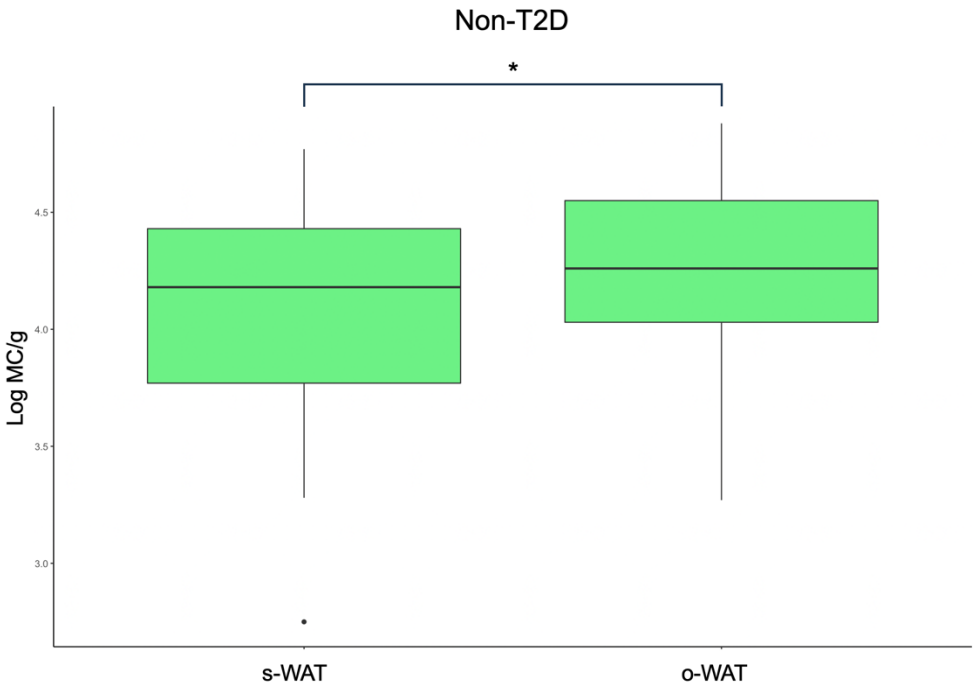

*Figure 4. Comparison of MC/g in white adipose tissue between anatomical locations in patients without type 2 diabetes (T2D). S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue), * (p-value < 0.05), n=41.*

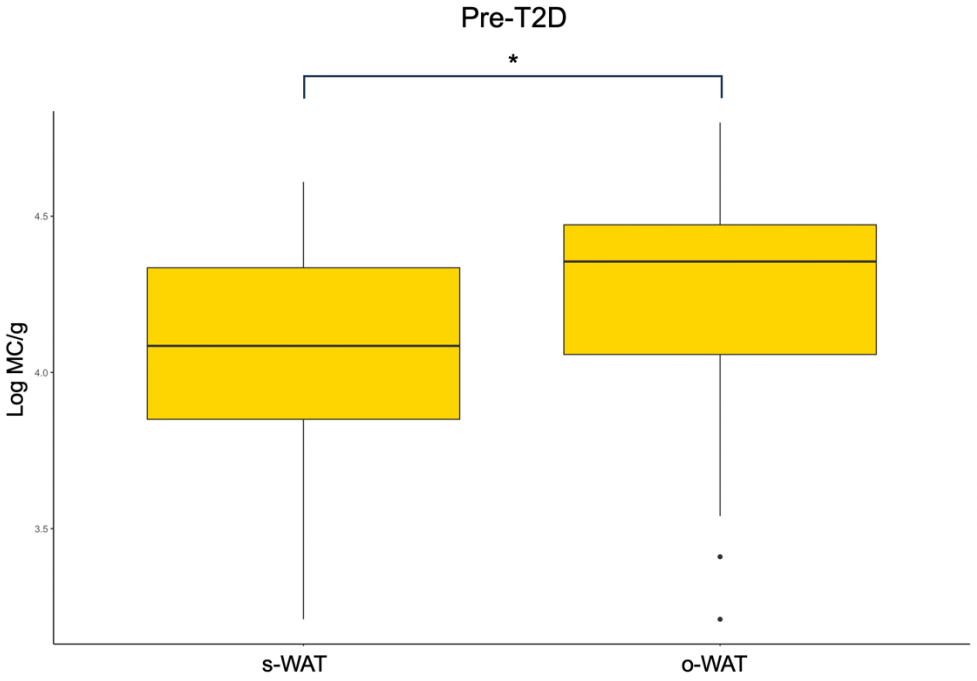

*Figure 5. Comparison of MC/g in white adipose tissue between anatomical locations in patients with pre- type 2 diabetes (T2D). S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue), * (p-value < 0.05), n=32.*

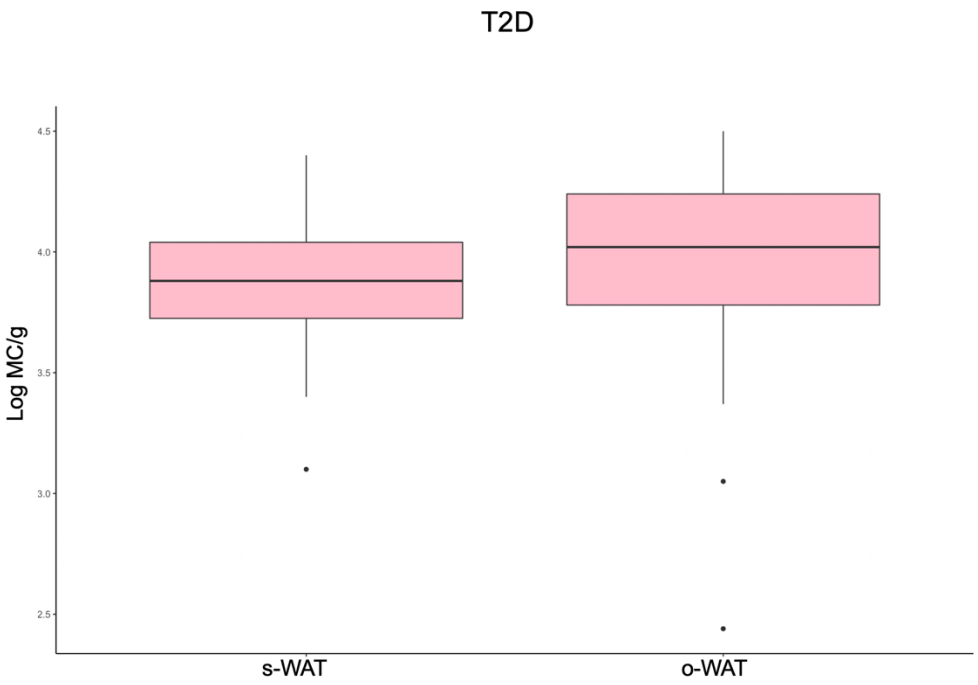

Figure 6. Comparison of MC/g in white adipose tissue between anatomical locations in patients with type 2 diabetes (T2D). S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue), n=27.

Mast cell count is a good predictor of T2D status

To identify the patterns between the measured variables and the three patient groups simplifying our high-dimensional data, we performed a principal component analysis (Figure 7). This statistical approach is used to summarize the internal structure of the data and visualize it. Noteworthy, in o-WAT and s-WAT, the number of mast cells was generally higher in the non-T2D group. The HbA1c variable was not incorporated in this and subsequent analyses, as it was employed to establish the groups.

Afterwards, a linear discriminant analysis (LDA) was performed to find the linear combinations of the original variables that can "split" the observations more efficiently in the transformed space when taking into account the levels of the "type 2 diabetes" variable (not, pre, yes) (Figure 8). Interestingly, the first linear combination (LD1) separates these observations well. Negative values of LD1 are more related to "No" values, while positive values are more related to "Pre" and "Yes". Then, the association between each variable and LD1 was explored (Table 4). There was a good correlation with age (0.50), waist-hip index (0.43) and mast cell count in o-WAT (-0.38). The negative correlation between mast cell count and LD1 means that patients with low values in LD1 tend to have a higher amount of mast cell counts. As the LD1 values of patients in the non-T2D group tended to be negative, this is consistent with our previous findings.

Finally, a random forest analysis was carried out. This approach is also used to measure the relevance of a variable to differentiate the (three) levels of the categorical variable "type 2 diabetes". This relevance is established by the "average reduction in the Gini index". Importantly, mast cell counts in o-WAT and s-WAT was the third and fifth most important variable, respectively (Figure 9). These are higher than some variables frequently studied in patients with T2D, including HDL, BMI and waist-hip index. Taken together, these results suggest that mast cells in adipose tissue, especially o-WAT, are closely related to T2D status. Given that this occurs in patients with a mild T2D condition, this phenomenon is expected to be more outstanding in patients with severe T2D and poor glycemic control.

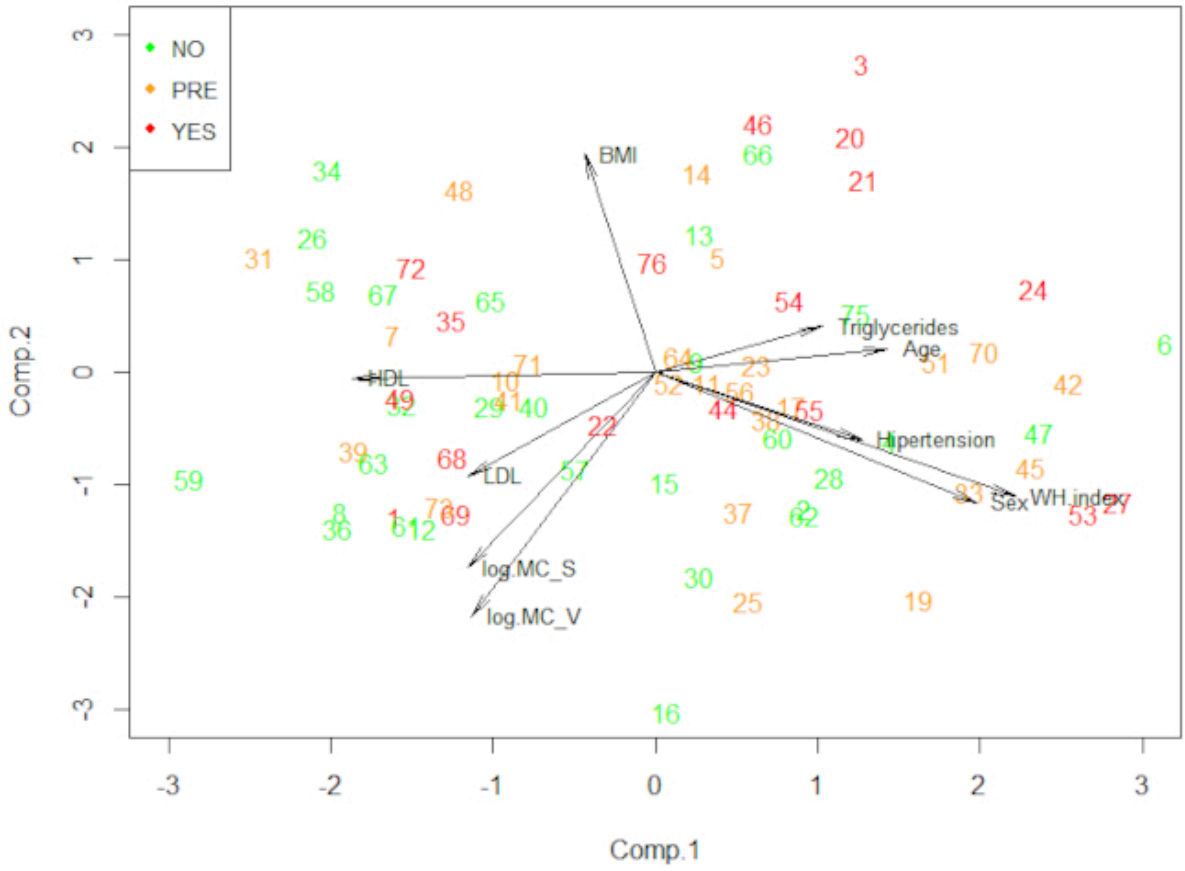

Figure 7. Principal component analysis (PCA). This approach reduces data dimension and identify conserved patterns. NO (patients without type 2 diabetes), PRE (patients with pre-type 2 diabetes), YES (patients with type 2 diabetes), BMI (Body Mass Index), MC_V (mast cells from omental white adipose tissue), MC_S (mast cells from subcutaneous white adipose tissue), WH index (waist hip index), n=100. The numbers are the patient identifiers inside the cohort.

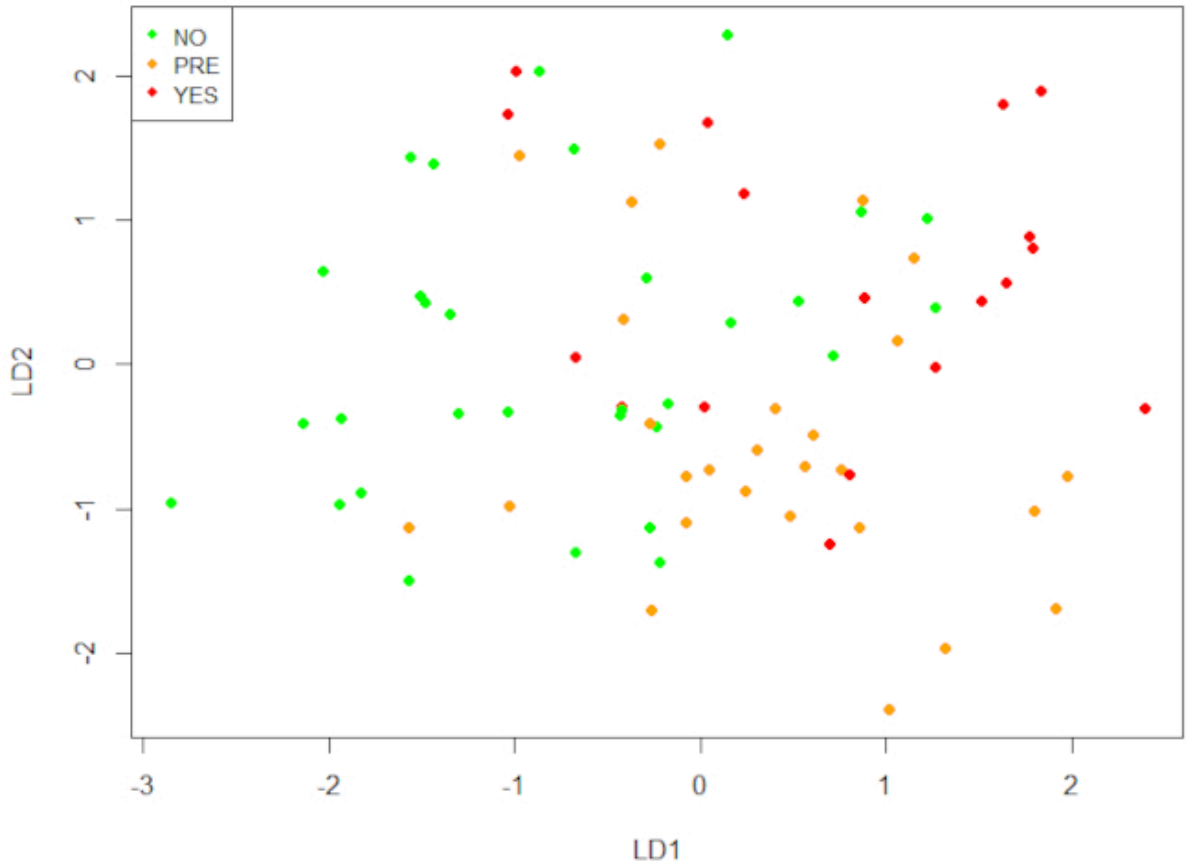

Figure 8. Linear discriminant analysis (LDA). This approach provides the linear combinations of variables that better separate observations. NO (patients without type 2 diabetes), PRE (patients with pre-type 2 diabetes), YES (patients with type 2 diabetes), LD1 (linear discriminant 1), LD2 (linear discriminant 2) n=100.

Table 4. Correlation of the original variables with LD1. The data employed here is from the big cohort (n=100). MC (mast cells), o-WAT (omental white adipose tissue), s-WAT (subcutaneous white adipose tissue) n=100.

Variable	**Correlation Coefficient**
Age	0.502
Waist-hip index	0.434
MC (o-WAT)	-0.380
HDL	-0.350

LDL	-0.319
Hipertension	0.300
Triglycerides	0.289
Sex (Male)	-0.280
MC (s-WAT)	-0.082
BMI	0.063

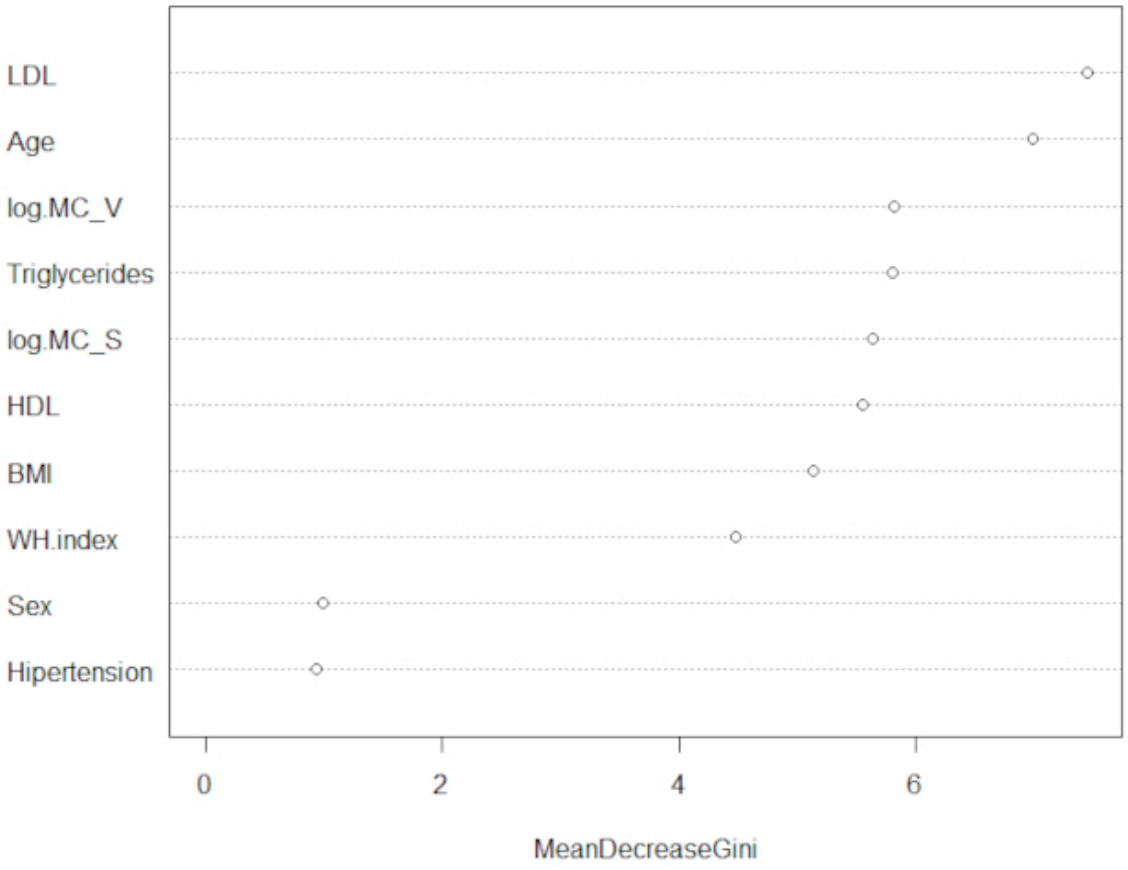

Figure 9. Random Forest Analysis. This approach calculates how each variable contributes to differentiate the three groups of patients. MC_V (mast cells in omental white adipose tissue), MC_S (mast cells in subcutaneous white adipose tissue), WH index (waist-hip index), n=100.

The surface abundance of CD45 on mast cells decreases in patients with T2D in o-WAT.

Figure 10 displays the relative abundance of CD45 on the surface of mast cells in o-WAT (n=96). Noteworthy, it looks like there is a trend that decrease towards T2D. Nevertheless, the only comparison statistically significant is between non-T2D and T2D. These results suggest that small changes in glycemic control have important consequences on CD45 surface abundance on mast cells in o-WAT. In s-WAT (Figure 11), the differences are not significant in any case. When compared o-WAT and s-WAT of the same patients in three groups, there is no significant difference between the CD45 amount of the mast cell surface (Figure 12Figure 14). However, in the T2D group, the difference was almost significant (p=0.072).

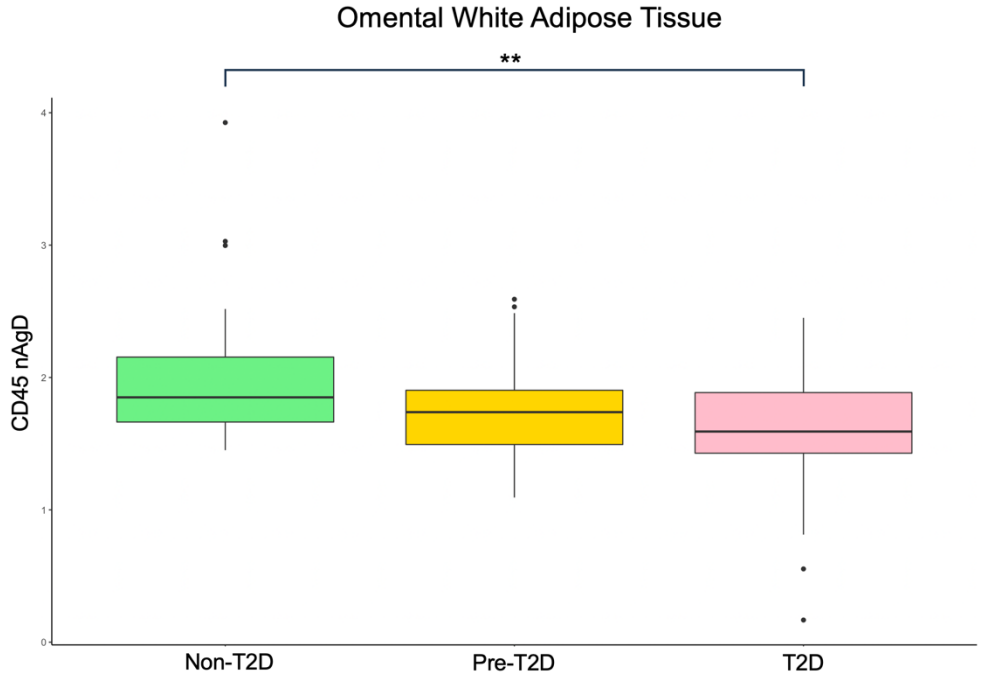

*Figure 10. Comparison of the normalized antigenic density (nAgD) of CD45 on the surface of mast cells in omental white adipose tissue depending on T2D (Type 2 Diabetes) status. ** (p value < 0.01), n=96.*

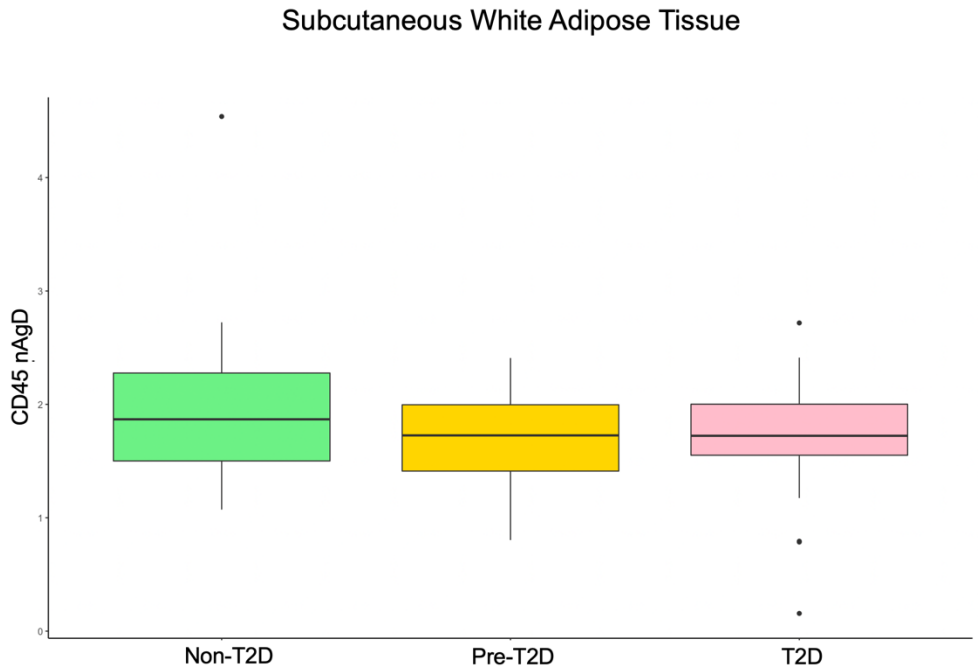

Figure 11. Comparison of the normalized antigenic density (nAgD) of CD45 on the surface of mast cells in subcutaneous white adipose tissue depending on T2D (Type 2 Diabetes) status, n=96.

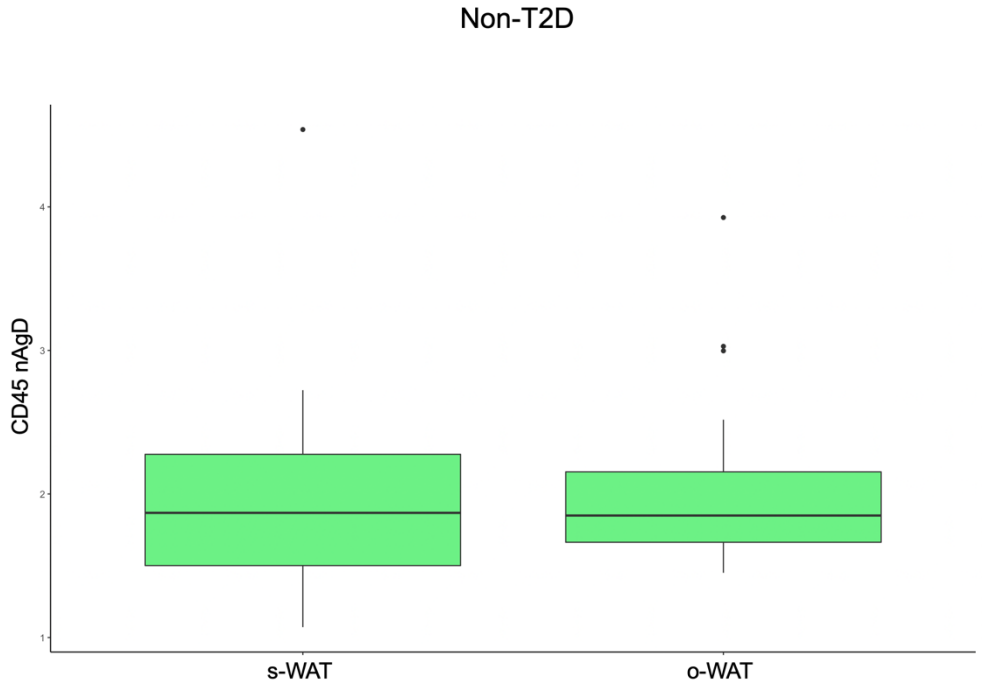

Figure 12. Comparison of the normalized antigenic density (nAgD) of CD45 on the surface of mast cells in patients without T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue) n=34.

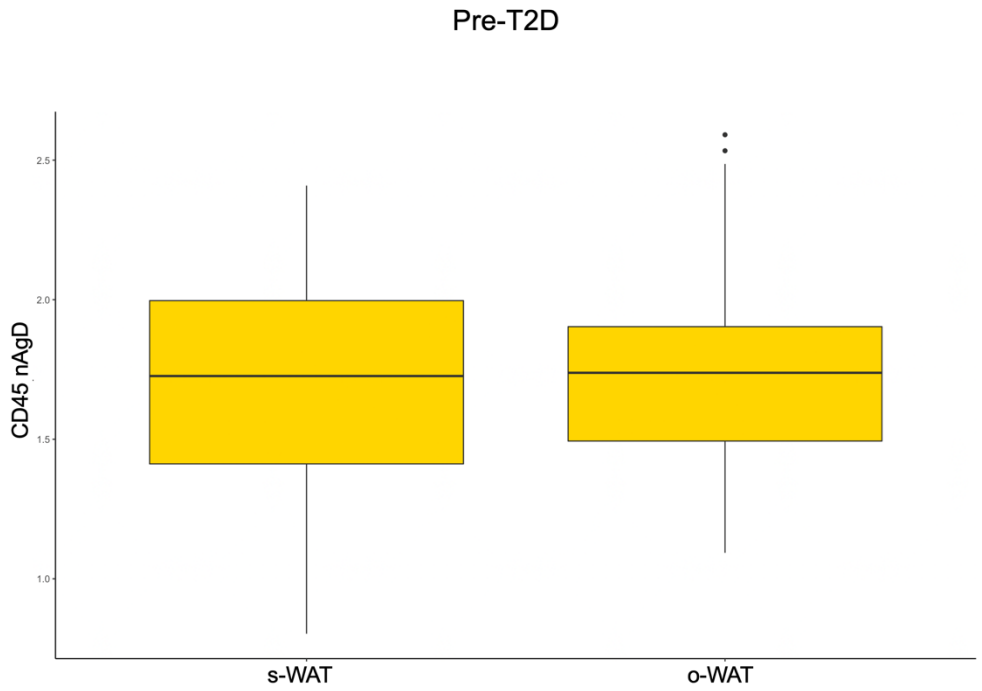

Figure 13. Comparison of the normalized antigenic density (nAgD) of CD45 on the surface of mast cells in patients with pre T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue) n=32.

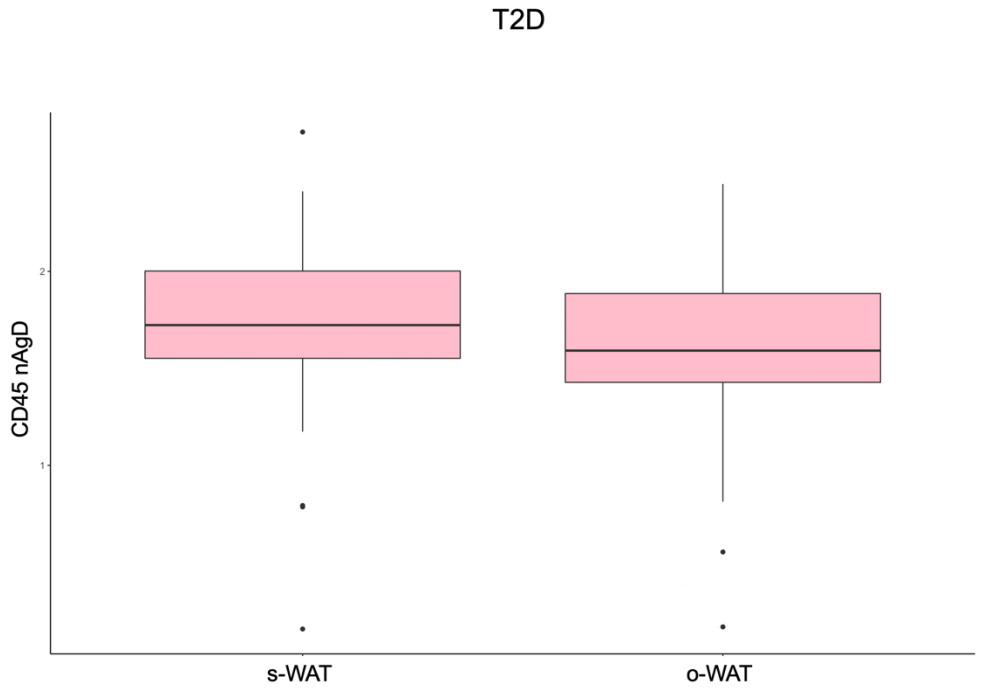

Figure 14. Comparison of the normalized antigenic density (nAgD) of CD45 on the surface of mast cells in patients with T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue) n=30.

The surface abundance of CD117 decreases in patients with T2D both in o-WAT and s-WAT

Figure 15 shows the surface abundance of CD117 on mast cells in o-WAT depending on the T2D status (n=96). There were statistically significant differences between non-T2D and T2D and among non-T2D and pre-T2D. Furthermore, in s-WAT (Figure 16), there was only a significant difference between non-T2D and T2D. Of note, the difference among o-WAT and s-WAT inside the same patient (Figure 17Figure 19) was significant just in pre-T2D and T2D. However, in non-T2D patients, the difference was nearly significant (0.071).

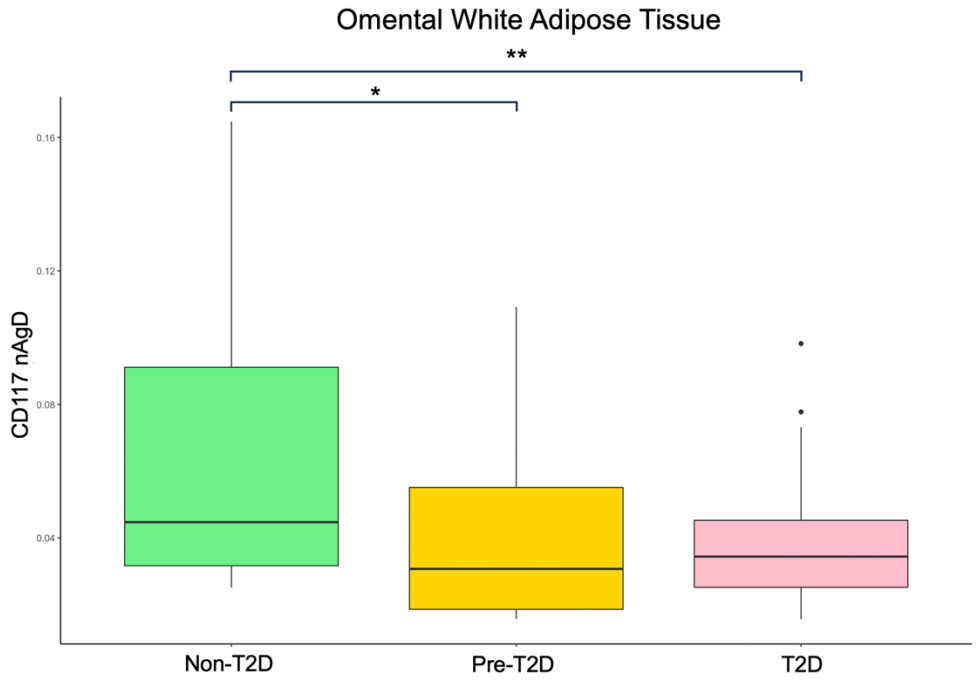

*Figure 15. Comparison of the normalized antigenic density (nAgD) of CD117 on the surface of mast cells in omental white adipose tissue depending on T2D (Type 2 Diabetes) status. * (p value < 0.05), ** (p value <0.01), n=96.*

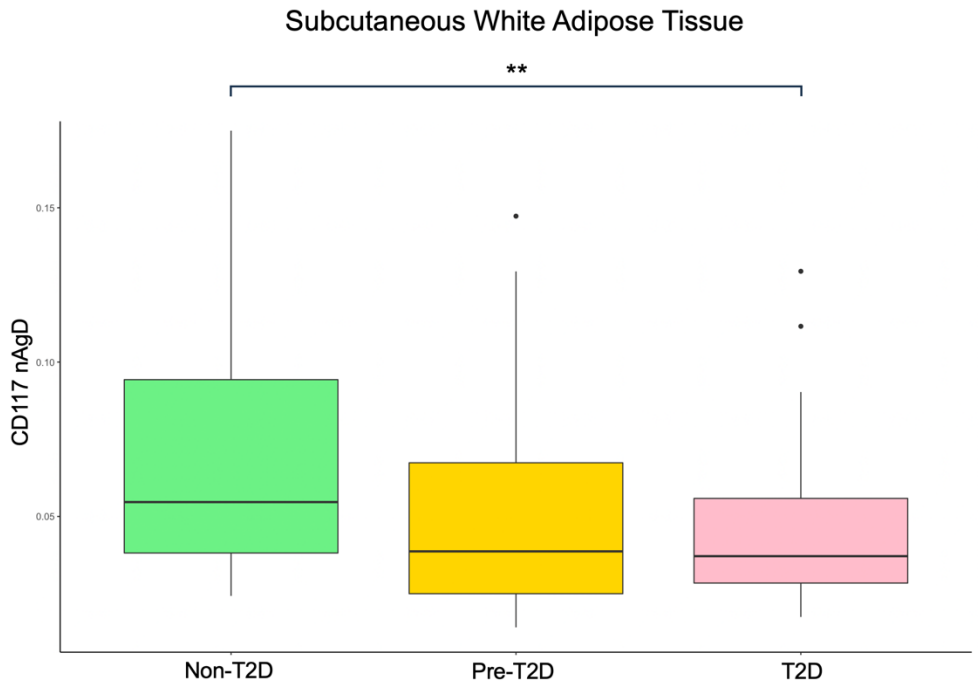

*Figure 16. Comparison of the normalized antigenic density (nAgD) of CD117 on the surface of mast cells in subcutaneous white adipose tissue depending on T2D (Type 2 Diabetes) status. ** (p value < 0.01) n=96.*

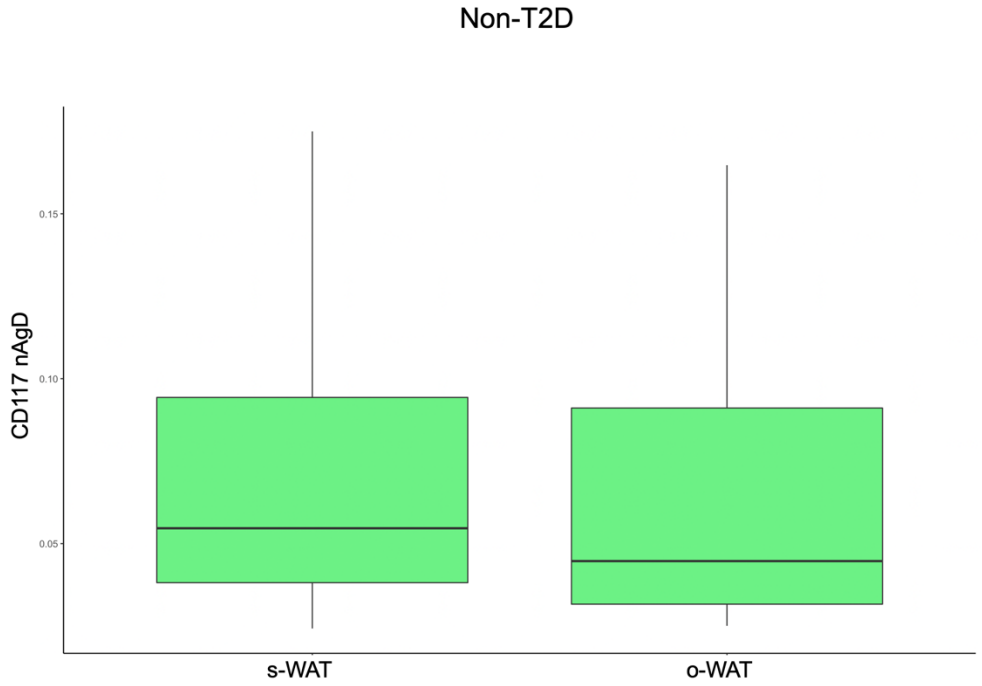

Figure 17. Comparison of the normalized antigenic density (nAgD) of CD117 on the surface of mast cells in patients without T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue) n=34.

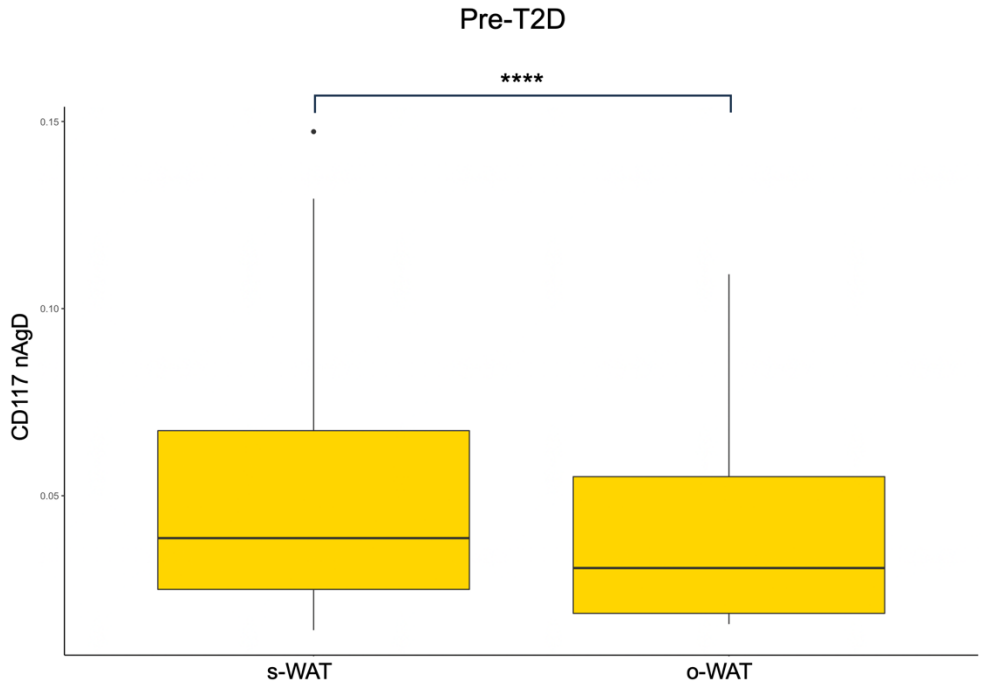

*Figure 18. Comparison of the normalized antigenic density (nAgD) of CD117 on the surface of mast cells in patients with pre T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue). **** (p value < 0.0001), n=32.*

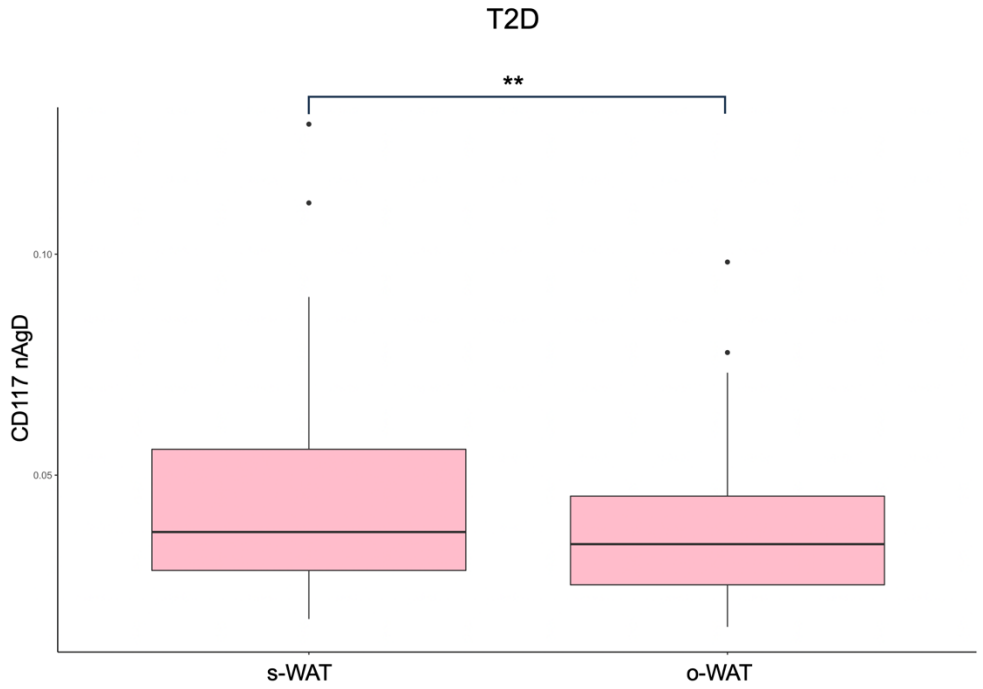

*Figure 19. Comparison of the normalized antigenic density (nAgD) of CD117 on the surface of mast cells in patients with T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue). **(p value < 0.01), n=30.*

The surface abundance of FceRI decreases in o-WAT in patients with type 2 diabetes

Figure 20 shows the surface expression of FceRI on mast cells in o-WAT (n=60). The only statistically significant comparison is between non-T2D and T2D. In contrast, there were no significant differences among groups in s-WAT (Figure 21). Nevertheless, when comparing o-WAT and s-WAT inside the same patient (Figure 22Figure 24), there were significant differences in all three groups. These data indicates that mast cells have higher surface abundance of FceRI in o-WAT than in s-WAT under normal conditions. Nonetheless, T2D has a greater effect on mast cell surface expression in o-WAT than in s-WAT.

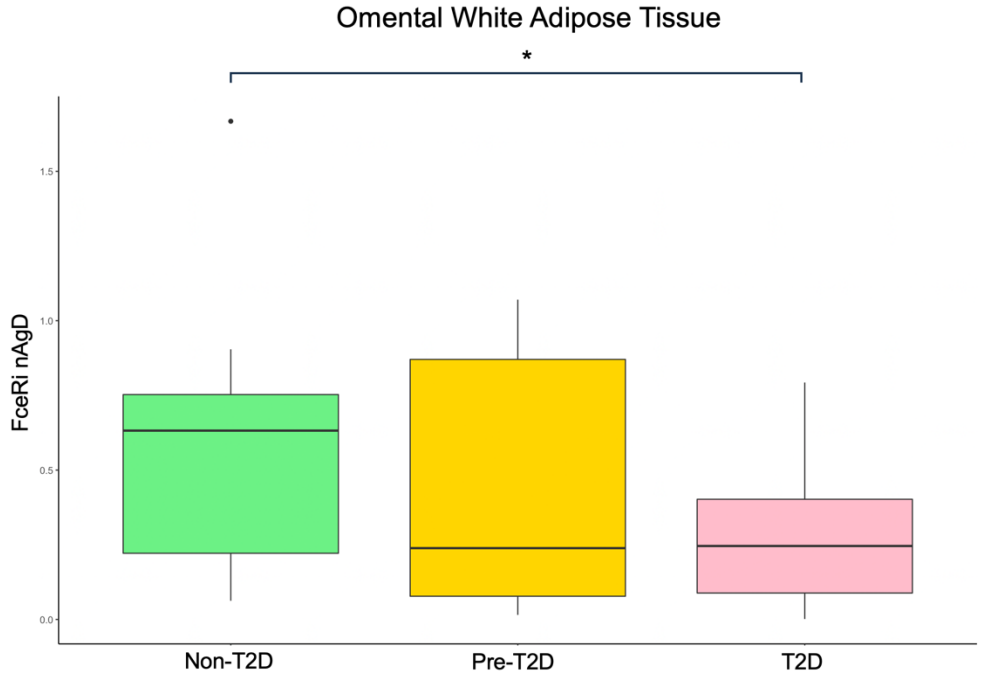

*Figure 20. Comparison of the normalized antigenic density (nAgD) of FceRI on the surface of mast cells in omental white adipose tissue depending on T2D (Type 2 Diabetes) status. * (p value < 0.05) n=60.*

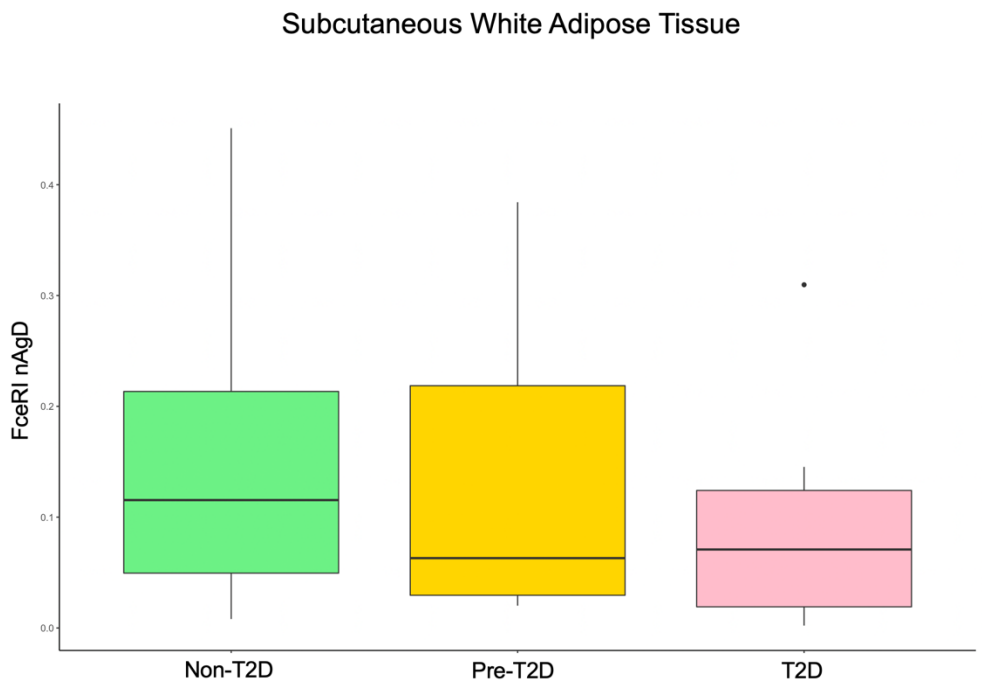

Figure 21. Comparison of the normalized antigenic density (nAgD) of FceRI on the surface of mast cells in subcutaneous white adipose tissue depending on T2D (Type 2 Diabetes) status n=60.

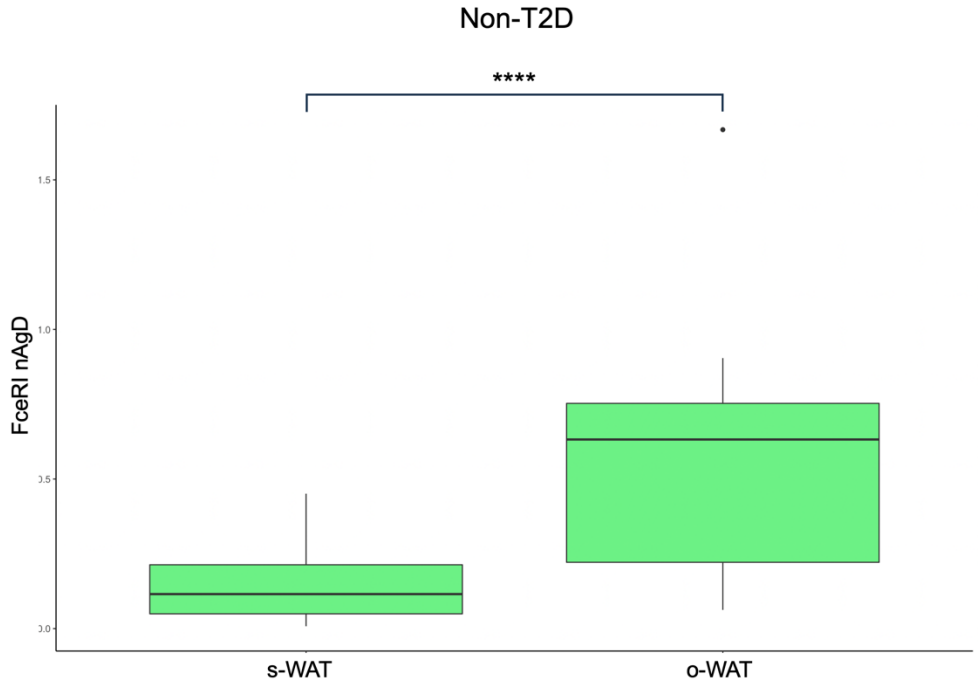

*Figure 22. Comparison of the normalized antigenic density (nAgD) of FceRI on the surface of mast cells in patients without T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue). **** (p value < 0.0001) n=23.*

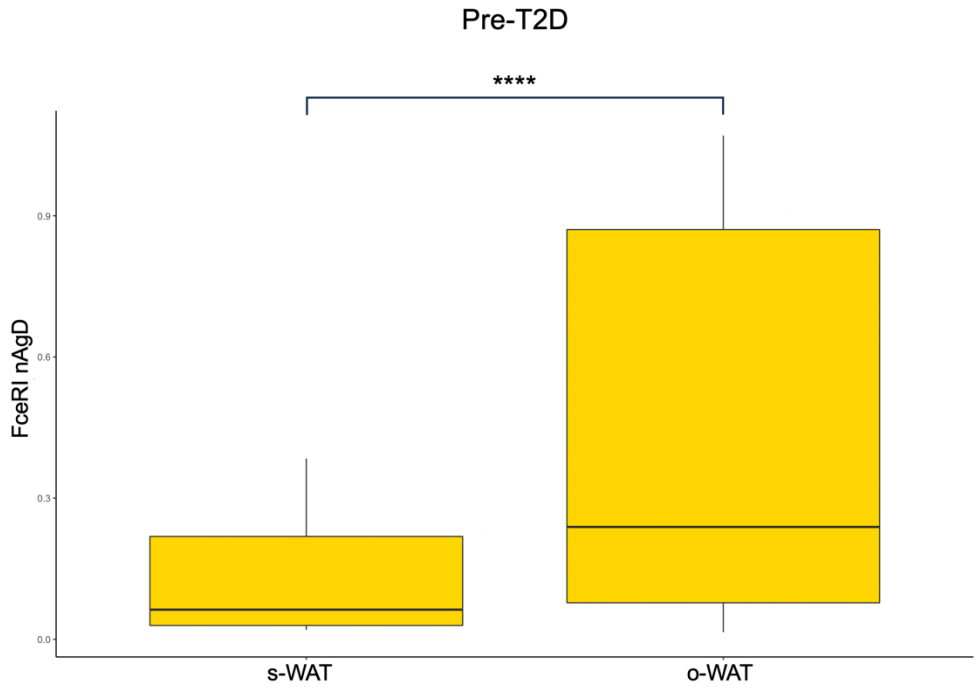

*Figure 23. Comparison of the normalized antigenic density (nAgD) of FceRI on the surface of mast cells in patients with pre T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue). **** (p value < 0.0001) n=21.*

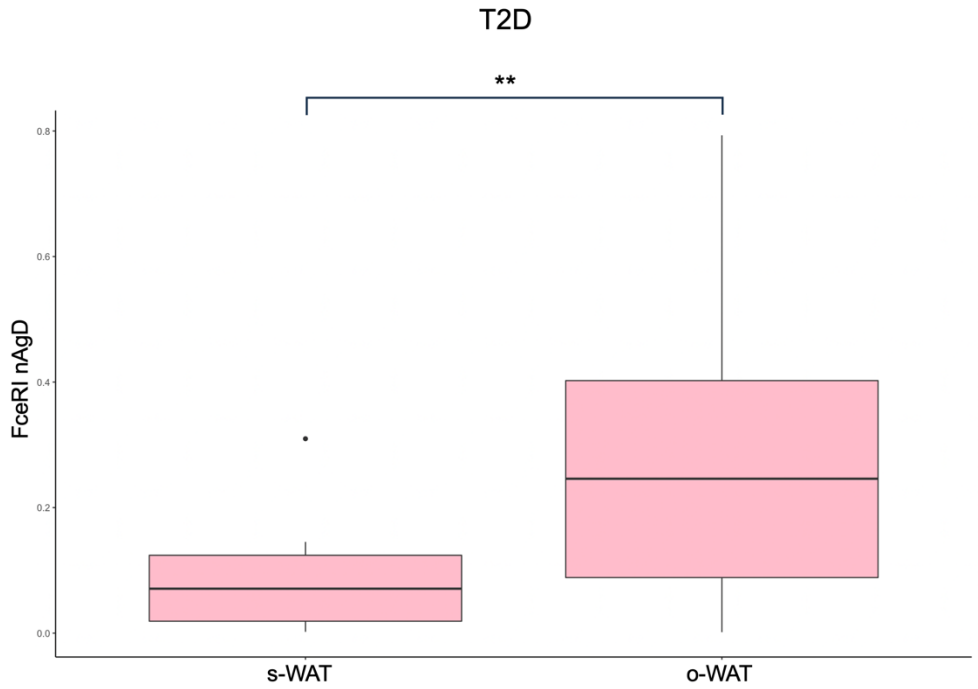

*Figure 24. Comparison of the normalized antigenic density (nAgD) of FceRI on the surface of mast cells in patients with T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue). ** (p value < 0.01) n=16.*

The surface expression of CD203c on mast cells decreases in o-WAT in patients with pre-T2D and T2D

Figure 25 shows the surface expression of CD203c on mast cells in o-WAT (n=27). Interestingly, there are significant differences among non-T2D and T2D and among non-T2D and pre-T2D. There were no significant differences between groups in s-WAT (Figure 26). These results indicate that surface expression of CD203c on mast cells in o-WAT is strongly influenced by changes in glycemic control. Noteworthy, the p-values obtained are very small taking into account the reduced sample size. This highlights the relevance of this phenomenon. Importantly, when comparing both adipose tissue locations inside the same patient the results were significant in all three groups.

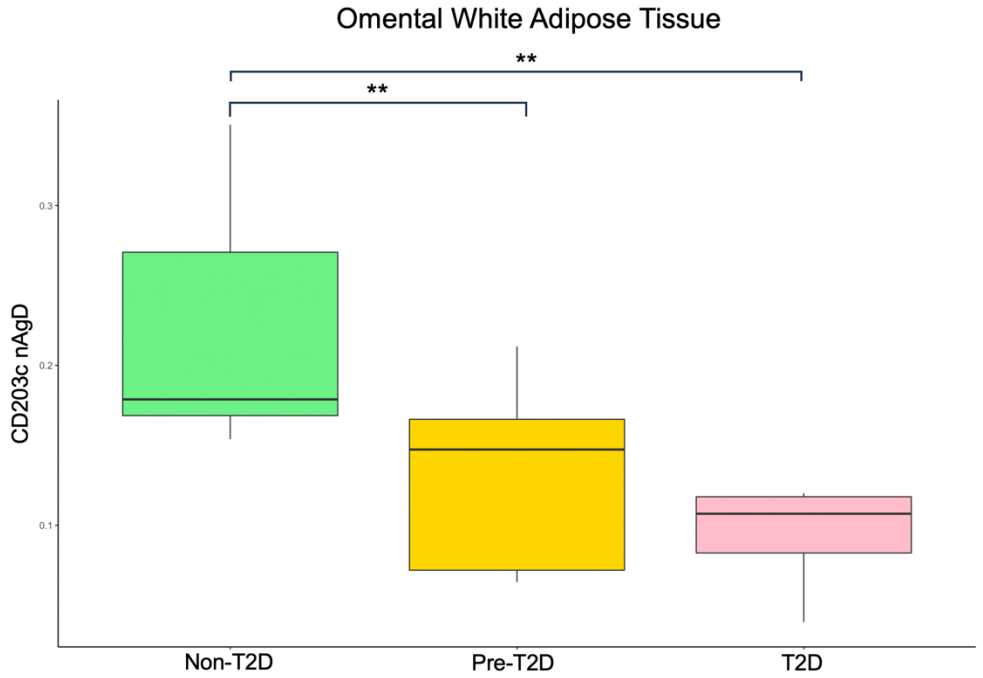

*Figure 25. Comparison of the normalized antigenic density (nAgD) of CD203c on the surface of mast cells in omental white adipose tissue depending on T2D (Type 2 Diabetes) status. ** (p value < 0.01) n=27.*

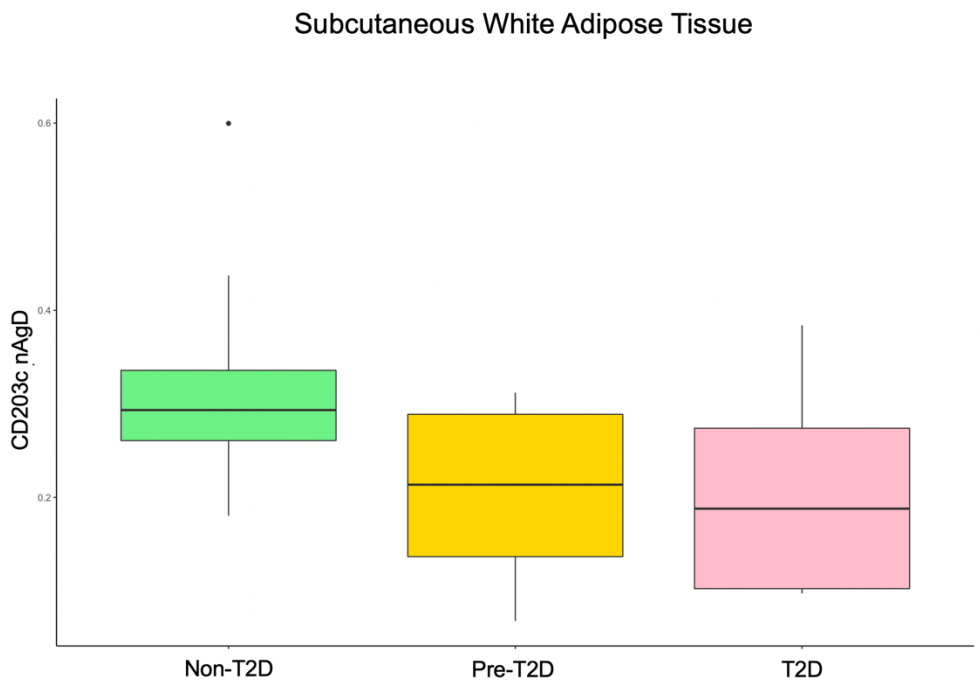

Figure 26. Comparison of the normalized antigenic density (nAgD) of CD203c on the surface of mast cells in subcutaneous white adipose tissue depending on T2D (Type 2 Diabetes) status n=27.

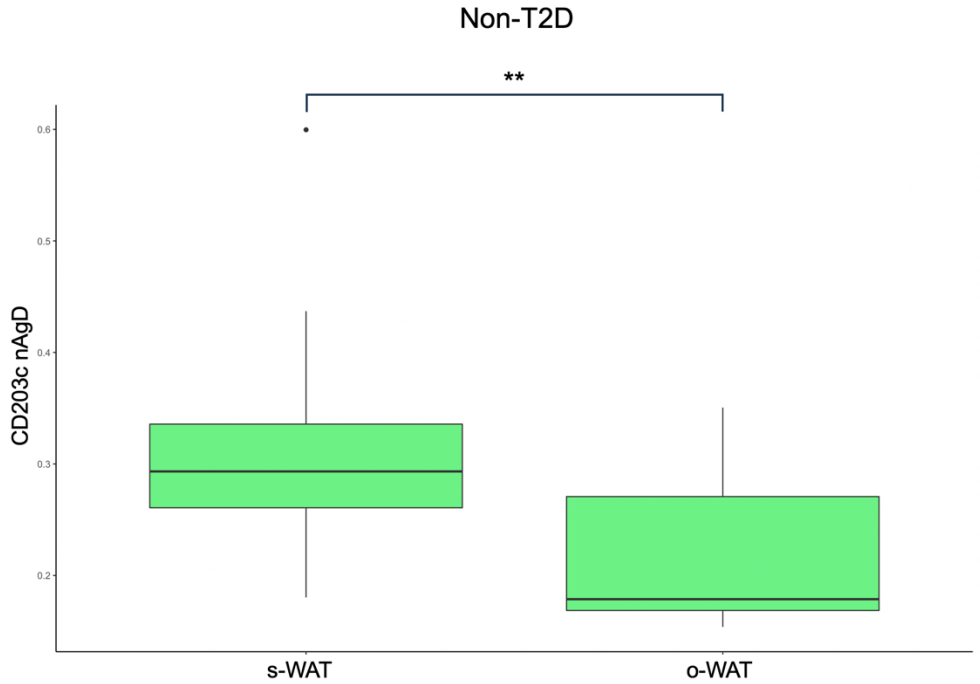

*Figure 27. Comparison of the normalized antigenic density (nAgD) of CD203c on the surface of mast cells in patients without T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue). **(p value < 0.01) n=10.*

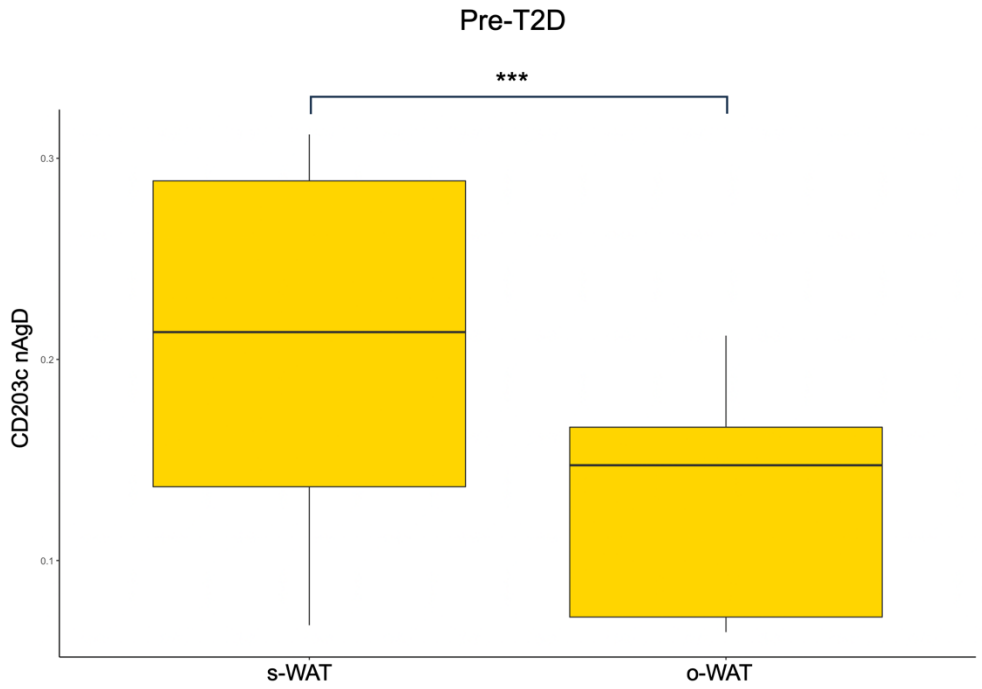

*Figure 28. Comparison of the normalized antigenic density (nAgD) of CD203c on the surface of mast cells in patients with pre T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue). ***(p value < 0.001) n=9.*

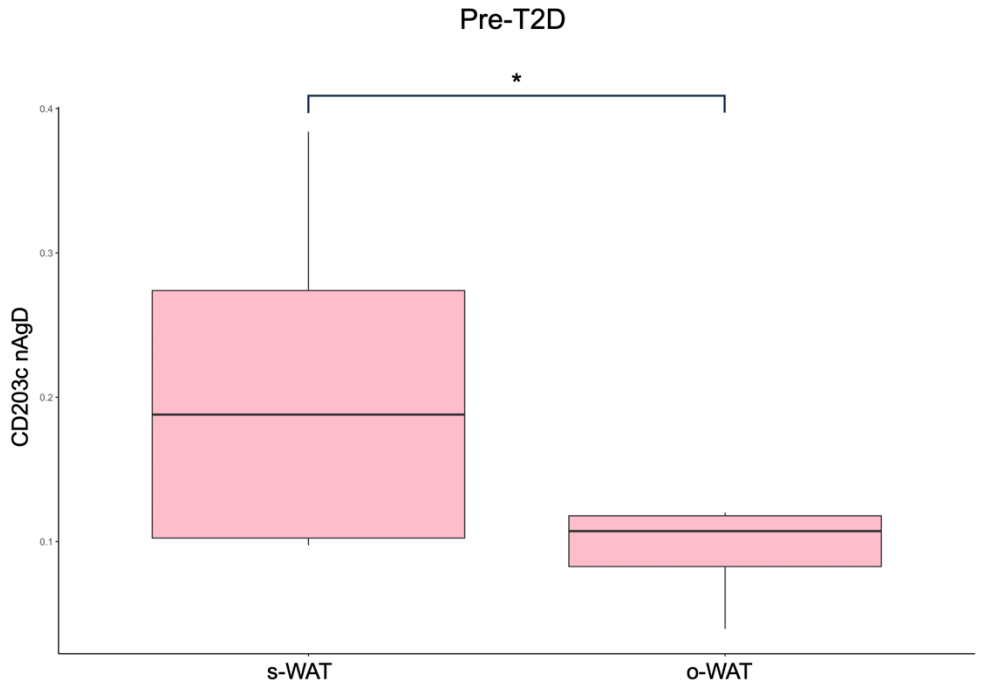

*Figure 29. Comparison of the normalized antigenic density (nAgD) of CD203c on the surface of mast cells in patients with T2D (Type 2 Diabetes) between different anatomical locations. S-WAT (subcutaneous white adipose tissue), o-WAT (omental white adipose tissue). *(p value < 0.05) n=8.*

Validation of the nAgD results through RT-qPCR

Mast cells are the only cells that CD117, FcεRI, and CD203c in adipose tissue. Therefore, RNA from whole adipose tissue was suitable for RT-qPCR (n = 60). Unfortunately, since all leukocytes in adipose tissue express CD45, it does not make sense to perform the RT-qPCR. Figure 30Figure 35 exhibit the gene expression of CD117, FcεRI and CD203c both in o-WAT and s-WAT. Importantly, the RT-qPCR assay showed the amount of mRNA normalized by the expression of two housekeeping genes (PPIA and RPS13). Nevertheless, flow cytometry quantifies the surface density of multiple proteins of interest in a specific cytometric population. Despite the two methods target different stages of gene expression, the RT-qPCR data for CD117, FcεRI and CD203c followed the same trends as the surface abundance analysis. Such reinforces the surface abundance data previously discussed.

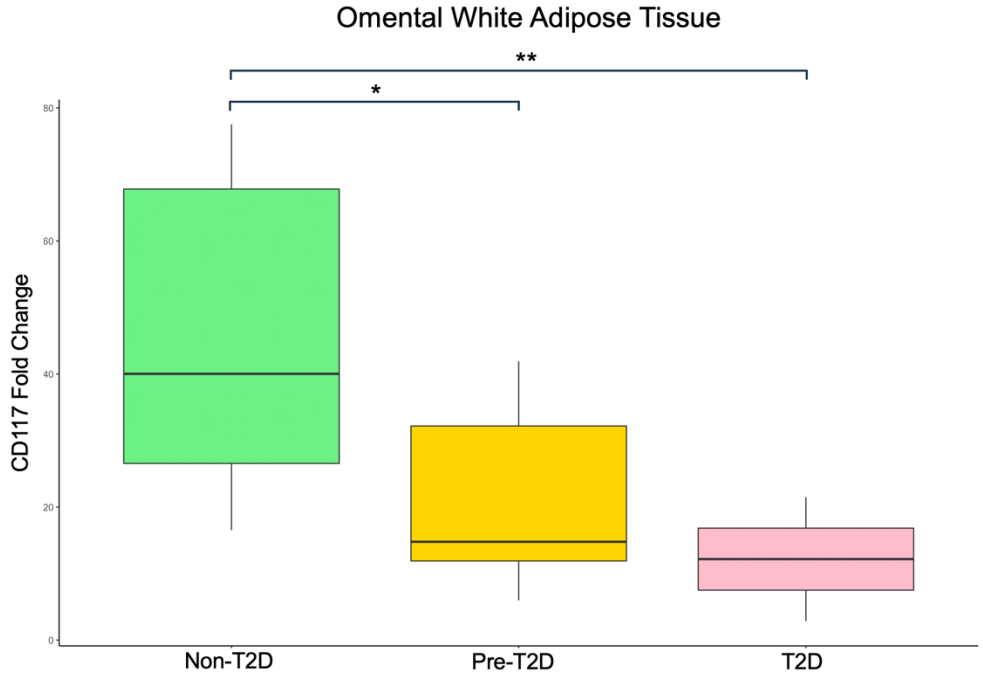

*Figure 30. Variation of CD117 fold change depending on the T2D diabetes status in omental white adipose tissue. T2D (type 2 diabetes). *(p value < 0.05), **(p value < 0.01) n=60.*

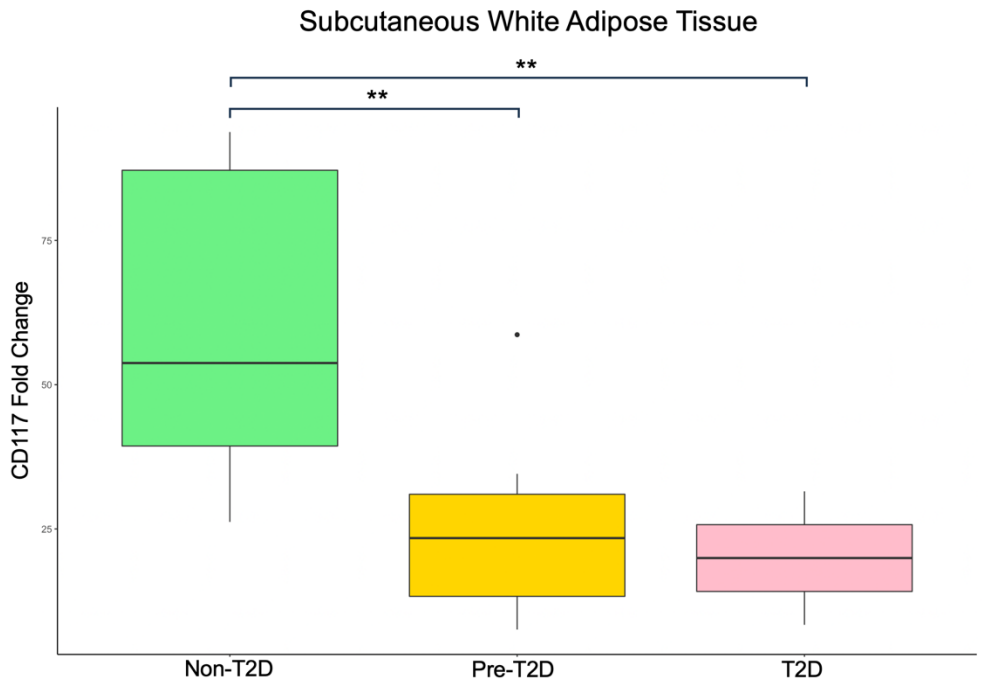

*Figure 31. Variation of CD117 fold change depending on the T2D diabetes status in subcutaneous white adipose tissue. T2D (type 2 diabetes). **(p value < 0.01) n=60.*

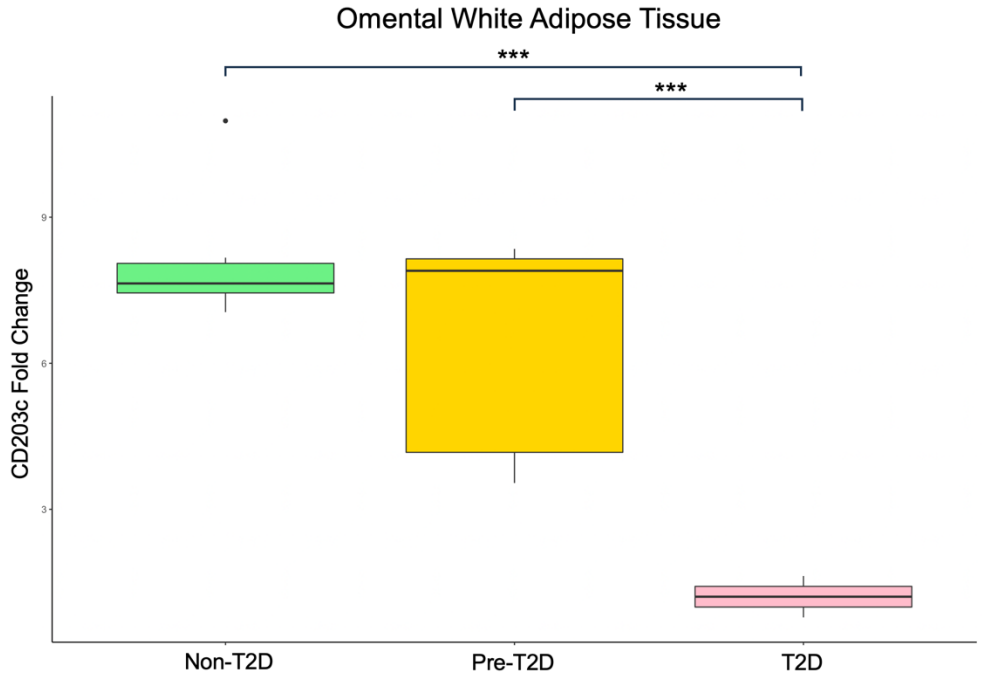

*Figure 32. Variation of CD203c fold change depending on the T2D diabetes status in omental white adipose tissue. T2D (type 2 diabetes). ***(p value < 0.001) n=60.*

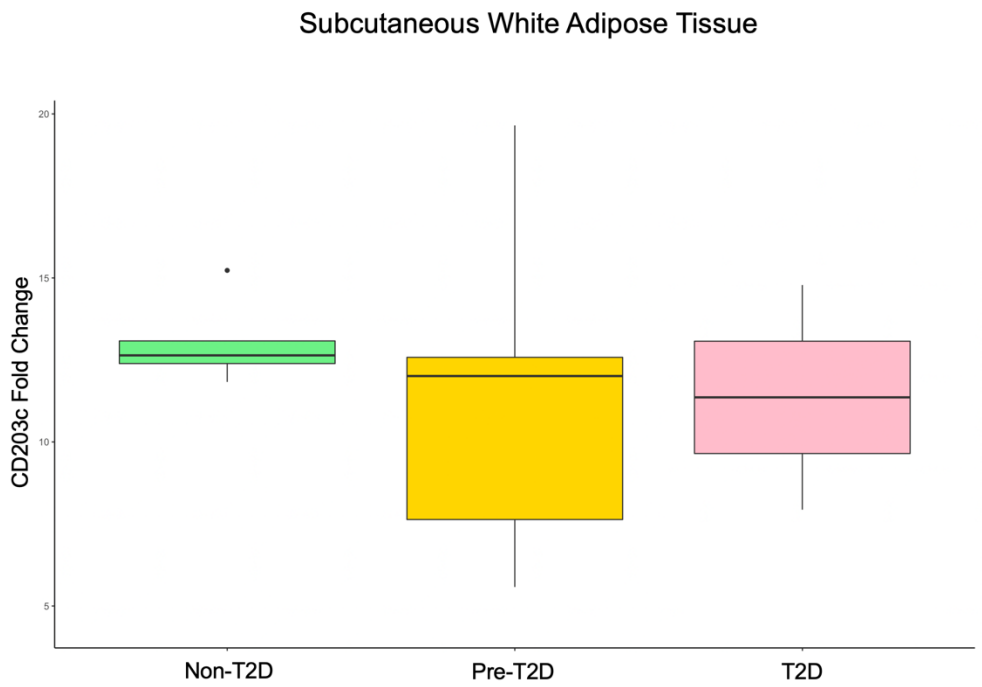

Figure 33. Variation of CD203c fold change depending on the T2D diabetes status in subcutaneous white adipose tissue. T2D (type 2 diabetes) n=60.

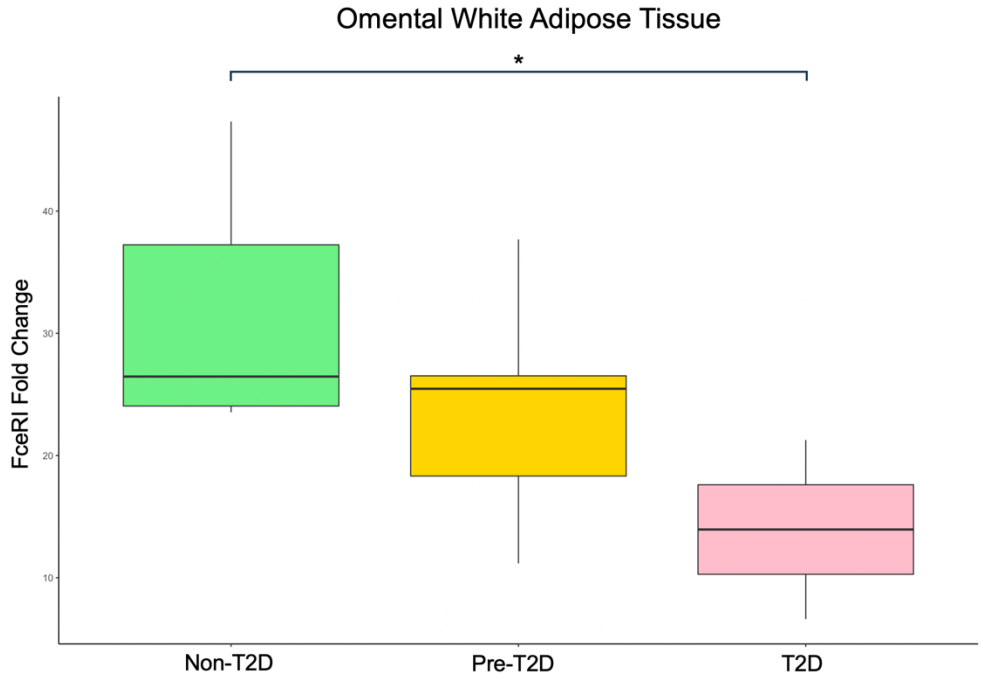

*Figure 34. Variation of FceRI fold change depending on the T2D diabetes status in omental white adipose tissue. T2D (type 2 diabetes). *(p value < 0.05) n=60.*

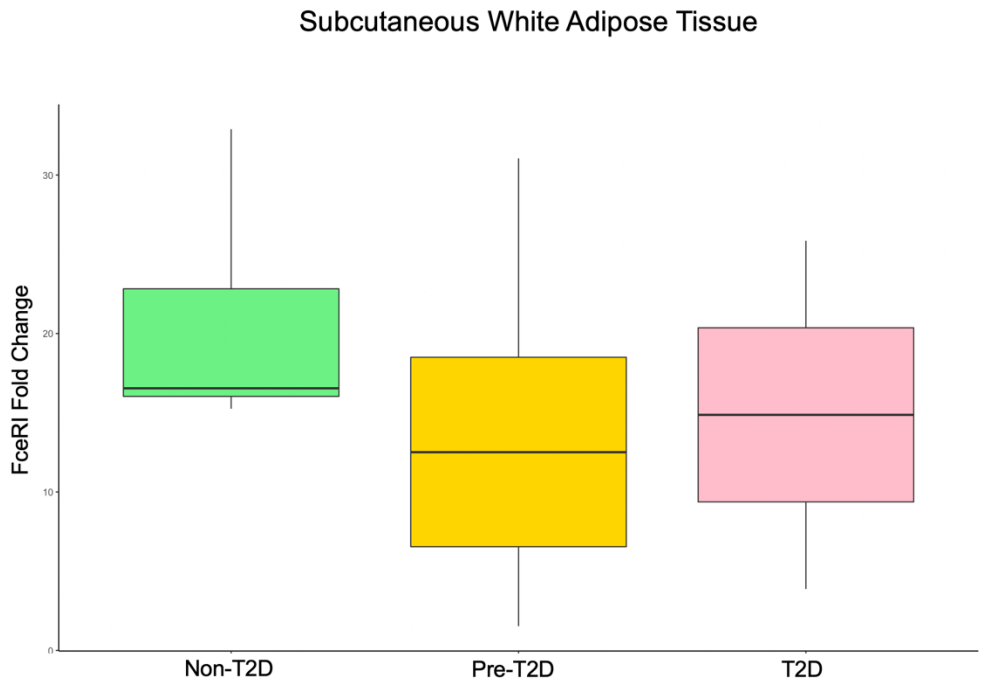

Figure 35. Variation of FceRI fold change depending on the T2D diabetes status in subcutaneous white adipose tissue. T2D (type 2 diabetes) n=60.

The surface abundance of CD45 on mast cells in o-WAT is a good predictor of T2D

We employed a principal component analysis (PCA) to identify patterns in this high-dimensional dataset. As displayed in Figure 36, patients in the non-T2D group appear in the left part of the graph. Then, a multiple regression analysis was performed to see whether there was a relationship between the variable HbA1c (which defines the T2D status) and the other variables studied. Importantly, significant p-values were obtained with age and mast cell CD45 surface abundance in o-WAT in the individual t-tests. Furthermore, the p-value for CD117 mast cells in o-WAT was nearly significant (p = 0.066).

Besides, a linear discriminant analysis was performed. This identifies the linear combinations of the original variables that provide a better separation of observations in the transformed space given the levels of the "type 2 diabetes" variable (no, pre, yes). Figure 37 shows that the first linear combination (LD1) is quite good separating the observations. The distribution of the patients across LD1 is the following: patients with non-T2D tend to have negative values, patients with pre-T2D have values around zero, and patients with T2D tend to have positive values. Correlations between each variable and LD1 are shown in Table 5. Importantly, a good correlation was observed with the surface abundance of CD45 on mast cells in o-WAT (-0.59), age (0.55) and surface abundance of CD117 on mast cells in o-WAT (-0.48).

Finally, we used random forest analysis to determine the relevance of each variable in differentiating the three groups. This relevance is determined by the average decrease in the Gini index. Figure 38 convincingly exhibits that the surface abundance of CD45 and CD117 on mast cells in o-WAT are the first and third most relevant variables respectively. These two were more relevant than variables typically measured in this type of patients including lipid metabolism (cholesterol, triglycerides, HDL and LDL) and BMI. These data indicates that there is a close relationship among mast cells and glycemic control in o-WAT.

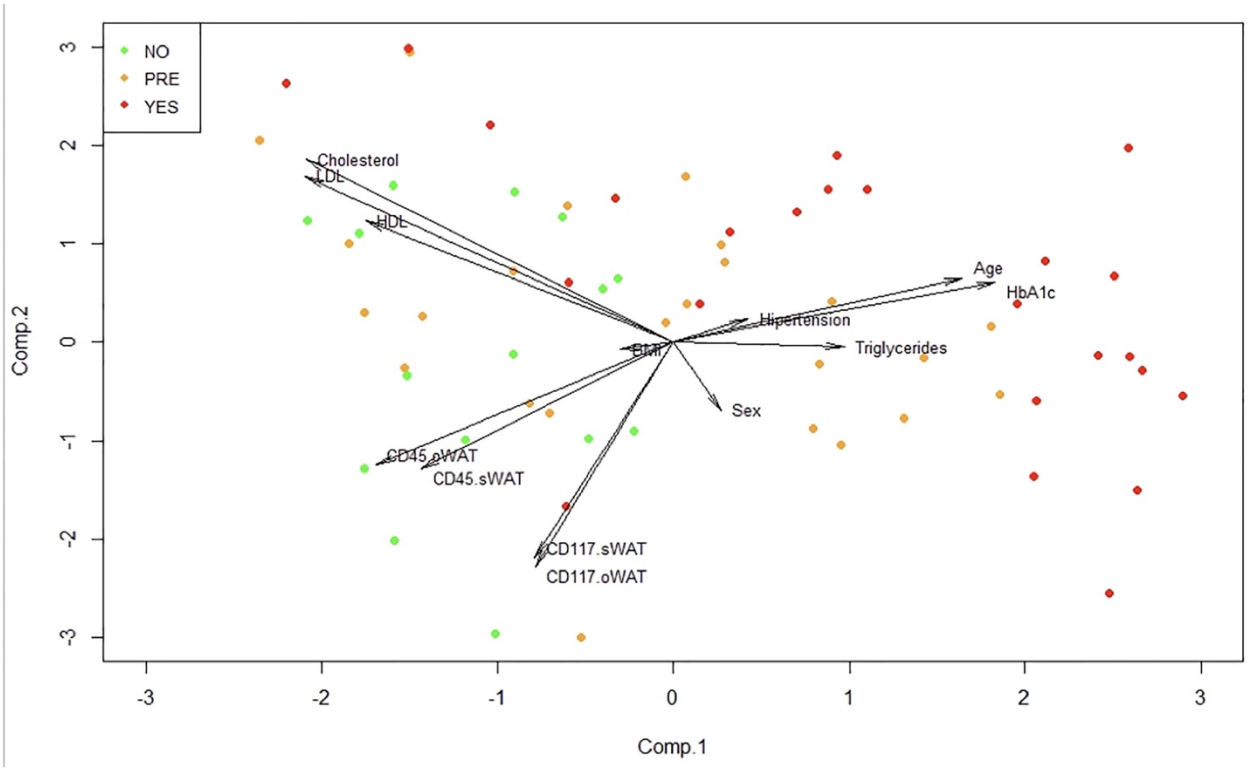

Figure 36. Principal component analysis (PCA). This approach reduces data dimension and identify conserved patterns. BMI (body mass index), oWAT (omental white adipose tissue), sWAT (subcutaneous white adipose tissue) Comp (component), NO (non-type 2 diabetes group), PRE (pre-type 2 diabetes group), YES (type 2 diabetes group), n=96.

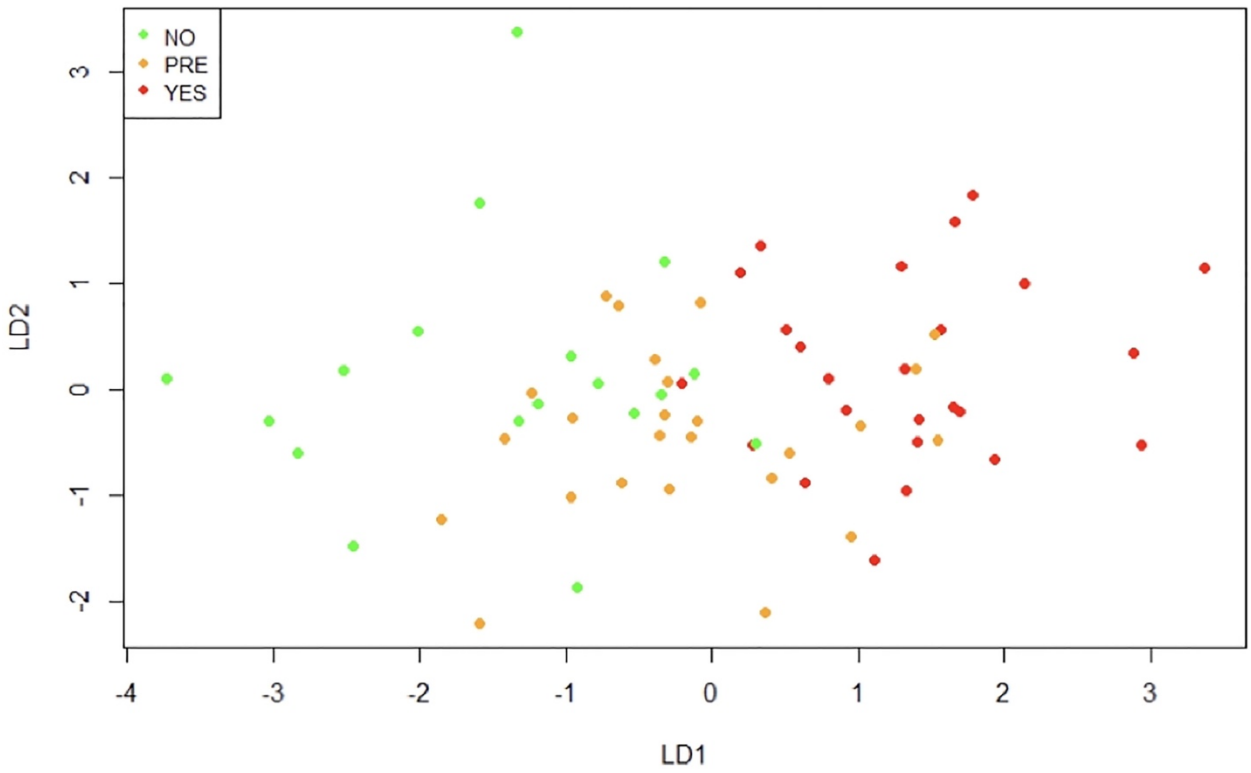

Figure 37. Linear discriminant analysis (LDA). This approach provides the linear combinations of variables that better separate observations. NO (non-type 2 diabetes group), PRE (pre-type 2 diabetes group), YES (type 2 diabetes group), LD1 (linear discriminant 1), LD2 (linear discriminant 2), n=96.

Table 5. Correlation of each variable with LD1. BMI (body mass index), o-WAT (omental white adipose tissue), s-WAT (subcutaneous white adipose tissue), LD1 (linear discriminant 1).

Variable	**Correlation with LD1**
CD45 o-WAT	-0.588
Age	0.547
CD117 o-WAT	-0.485
CD117 s-WAT	-0.412
LDL	-0.393
CD45 s-WAT	-0.370

Cholesterol	-0.337
BMI	-0.294
Triglycerides	0.257
Hypertension	0.204
HDL	-0.125

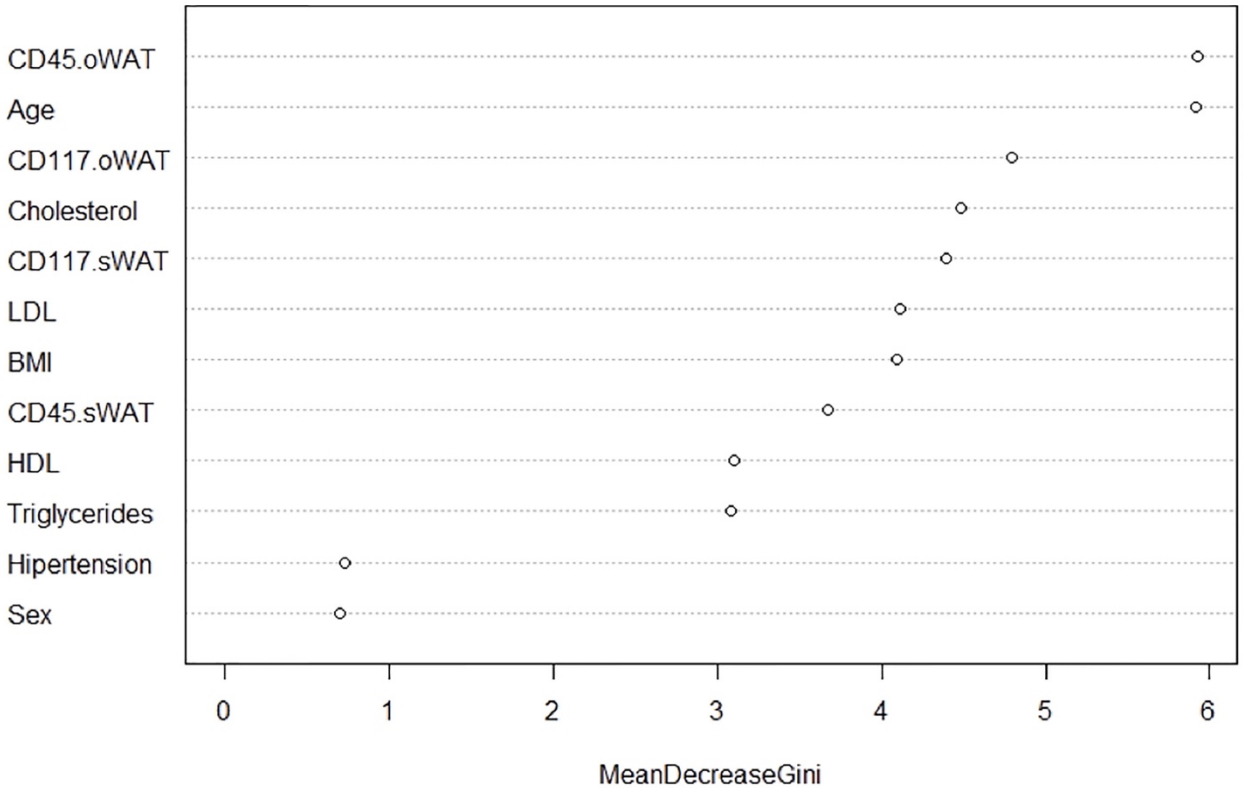

Figure 38. Random Forests Analysis. This approach calculates how each variable contributes to differentiate the three groups of patients. BMI (body mass index), o-WAT (omental white adipose tissue), s-WAT (subcutaneous white adipose tissue).

Discussion

Mast cells are well-known for their roles in inflammation and allergy, raising the question of whether they also contribute to obesity and T2D.

A prior study reported an increase in mast cell numbers in adipose tissue in patients with T2D (90). However, this study relied on histological slides and normalized mast cell numbers with the surface of fibrosis in tissue biopsies, which lacked precision. Importantly, mast cells are not confined to fibrotic tissue and are frequently found around blood vessels (22). To address this, the present study employed a more accurate cell-counting method by digesting whole samples (2-2.5g) and performing a flow cytometry.

Other studies in murine models found that cromolyn, a mast cell stabilizer, reduced obesity, adipose tissue fibrosis, and promoted insulin sensitivity (80,91). Although cromolyn is used to treat asthma, its mechanism of action remains poorly understood (92). Interestingly, mucosal mast cells respond to cromolyn, while connective tissue mast cells do not (92,93). Furthermore, TNFα production showed no significant difference between wild-type and mast cell-deficient mice, but cromolyn inhibited TNFα production in both groups in a dose-dependent manner, suggesting that cromolyn also target other immune cells (92).

Genetic ablation of CD117, a critical receptor for mast cell development, protected mice from weight gain and insulin resistance (80). However, further experiments showed that it was CD117 ablation, not mast cell depletion, what improved the metabolic profile (81). This occurs because CD117 is also important in the development of other immune cells including T lymphocytes (94), B lymphocytes (95), and neutrophils (96). Despite various genetic models exploring the effects of mast cell absence in white adipose tissue (WAT), current evidence does not support their deleterious role in obesity and insulin resistance (97,98). The problem of these models is that they primarily focused on the immunological function of mast cells (as cells with inflammatory potential). However, mast cells have a deeper relationship with adipose tissue. Mast cells are present in WAT during the embryonic stage, where they differentiate under the influence of the local microenvironment (99,100). They contribute to WAT homeostasis by interacting with adipokines, lipoproteins, and stromal cells (98,101,102). Besides, they are relevant in physiological responses of WAT. In cold conditions, mast cell numbers increase in WAT, promoting the browning of WAT in response to norepinephrine (103,104). They also play a vital role in adipose tissue expansion by promoting angiogenesis (22) and glucose-dependent adipogenesis (77,78), contributing to metabolically healthy adipose tissue expansion.

In a nutshell, mast cells are not central players driving adipose tissue inflammation in patients with T2D. BMI remains similar irrespective of T2D status, indicating that the issue may be related to the mechanism of adipose tissue expansion.

When adipocytes expand in response to increased nutrient availability, oxygen diffusion decreases, causing mild hypoxia that triggers angiogenesis (105). Angiogenesis is impaired in

patients with T2D (73), exacerbating hypoxic stress and triggering inflammation and fibrosis (23,63,66,105,106). Mast cells sense this hypoxic condition through reactive oxygen species (ROS) and hypoxia-inducible factor 1α (HIF1α) production (22). To promote angiogenesis, they release pro-angiogenic factors (VEGF, FGF, TGF-beta), proteases to remodel the ECM, histamine to increase vascular permeability, and heparin (22). They also downregulate pro-inflammatory cytokine production in response to hypoxia (107) and interact with endothelial cells to promote their proliferation and release of angiogenic factors (108). Noteworthy, in the absence of mast cells, adipose tissue is less vascularized (109).

Furthermore, mast cells contribute to foam cell formation and pre-adipocyte to adipocyte differentiation (22,77,78,110), affecting the adipose tissue's capacity to uptake and store lipids. The decrease in the number of mast cells reduce this capacity of lipid accumulation in adipose tissue. This results in increased free fatty acids, causing hyperlipidemia, which activates TLR2 and TLR4 on macrophages, promoting inflammation (23,106), as well as gluconeogenesis in the liver, leading to hyperglycemia (111) and hepatic steatosis (64). Hyperlipidemia is also an indicator of a poor prognosis for the development of cardiovascular diseases (112).

There is limited research on mast cells in WAT, with most studies relying on murine models. Unfortunately, there are important differences between mouse and humans when it comes to WAT. In humans. Humans tend to expand WAT in omental and mesenteric depots while mice expand WAT in gonadal depots (56,59). Most sexual hormones are liposoluble and WAT can be greatly influenced by them (113). Therefore, the proximity between the gonads and adipose tissue in mice generates a different microenvironment. This limitation is not frequently discussed but has important implications for the translation of mice results to humans. Besides, in mice, WAT fibrosis impairs tissue function, but the situation is more complex in humans. Fibrosis in human s-WAT is associated with a pathological metabolic profile, larger adipocytes, and less weight loss after bariatric surgery (114). In contrast, fibrosis in omental WAT (o-WAT) is linked to smaller adipocytes (115,116), providing a better metabolic profile (115,116). Mast cells are abundant in fibrotic tissue, where they can either produce or degrade collagen depending on the molecular context (117). In adipose tissue fibrosis, their role remains unclear (117). However, in the liver, mast cells have an antifibrotic role (118).

Previous research has reported three key aspects of adipose tissue physiology after weight loss induced by bariatric surgery (119). Firstly, extensive tissue remodeling occurs, increasing insulin sensitivity. Secondly, pre-adipocyte numbers increase, and thirdly, adipocyte size decreases. These changes suggest a remodeling process leading to normalized glucose metabolism. Bariatric surgery also reduces inflammation in both o-WAT and s-WAT, including a substantial decrease in neutrophil numbers (119). Importantly, after bariatric surgery, mast cell numbers increase 10 times in o-WAT and 4 times in s-WAT (119). Consequently, as mast cells increase and promote adipogenesis, they likely play a major role in adipose tissue remodeling.

A study by Goldstein et al. (120) demonstrated that in patients with obesity, a higher number of mast cells in o-WAT is associated with a lower cardiometabolic risk. Moreover, more mast cells in o-WAT correlate with greater weight loss after bariatric surgery. These findings suggest that

mast cells have a close relationship with WAT physiology and play an essential role in it (22,77,78,107,110). These findings indicated that T2D influenced mast cells, although the specific phenotypic changes remained unclear.

Mast cells undergo extensive post-transcriptional and post-translational regulation (75,121). Among these, they can sequester membrane receptors intracellularly (122–125). For instance, CD203c is stored in the membrane of granules, and it is translocated to the plasma membrane after granule release (126,127). Traditional techniques like RT-PCR and Western Blot may overestimate the functional amount of certain membrane proteins on mast cells. This work presents a flow cytometry-based approach that detects mast cells and their surface expression of CD45, CD117, CD203c, and FceRI. This technique specifically detects membrane-bound proteins, providing a more accurate assessment of biologically active receptors. It has advantages over traditional methods, as it analyzes mast cells in the whole stromal vascular fraction and is not influenced by cell numbers. Furthermore, it saves time and resources by eliminating the need for mast cell purification. Similar technology has been employed on mast cells previously (127) and used for diagnosing certain pathologies, including chronic granulomatous disease (128,129). The use of the internal control also addresses potential errors introduced by flow cytometer laser oscillations, enhancing result accuracy and reproducibility.

Our research reveals phenotypic changes in mast cells in patients with T2D, particularly in o-WAT. The reduced surface expression of CD117 in patients with T2D suggests a decline in mast cell survival and activatory signals. Decreased plasma levels of stem cell factor, which binds CD117, are associated with T2D and cardiometabolic diseases (130,131), further contributing to mast cell death and reduced numbers.

Reduced surface expression of FceRI on mast cells in patients with T2D decreases their activation potential via IgE-antigen binding, as crosslinking of at least two IgE-FceRI complexes is required for activation (132). IgE binding to FceRI also promotes mast cell survival (133,134), compounding the decrease in mast cell numbers.

The reduced surface expression of CD45, a tyrosine phosphatase regulating the activation of Src family kinases, suggests a decline in mast cell effector function (135). This decline likely affects various mast cell interactions with other cells in the microenvironment, including the promotion of pre-adipocyte to adipocyte differentiation, lipid uptake by macrophages and their differentiation into foam cells, and angiogenesis.
Finally, a sharp decrease in surface expression of CD203c indicates reduced secretory activity in mast cells, further impairing their function in adipose tissue.

Conclusions

- The number of adipose tissue mast cells decreases in patients with T2D both in o-WAT and in s-WAT. However, the decrease is more significant in o-WAT.

- The surface abundance of CD117 on adipose tissue mast cells decreases in patients with T2D both in o-WAT and s-WAT.
- The surface abundance of CD45, CD203c, and FceRI on adipose tissue mast cells decreases in the o-WAT of patients with T2D but not in the s-WAT.

Chapter 2: Innate immune cells as important players of the tumor microenvironment in colorectal cancer

Introduction

Colorectal cancer (CRC) is the fourth most common cancer worldwide and the second leading cause of cancer-related deaths (136). The risk factors for CRC include age (especially after 50 years old), family history of CRC or inherited genetic conditions (such as Lynch syndrome or familial adenomatous polyposis), personal history of inflammatory bowel disease, smoking, obesity, lack of physical exercise, and a diet high in processed meats and low in fiber (137).

Since adenocarcinoma accounts for more than 90% of all CRC cases (138), in the present study CRC will always refer to colorectal adenocarcinoma. If CRC is detected in the early stages, the chances of successful treatment and improved survival rates are significantly higher (139). However, approximately half of CRC cases are diagnosed at advanced stages, which greatly reduces the chances of successful treatment and contributes to the high mortality rate (140). The principal cause of death in CRC patients is metastasis, which frequently occurs in the liver and lungs (141). Due to the high incidence and mortality rates of CRC, high income countries have implemented screening programs to detect CRC early and improve patient outcomes. These programs include fecal blood testing every 2 or 3 years, colonoscopy every 10 years, and the use of imaging techniques such as computed tomography scans and magnetic resonance imaging for further evaluation of suspicious lesions or metastases (142). These programs had a huge success and reduced mortality rates 30% (143). However, despite the progress in early detection and treatment options for CRC, the complex developmental mechanisms of colorectal cancer continue to present challenges in effectively treating the disease (144).

Molecular aspects of colorectal cancer

CRC is a genetically heterogeneous disease in which a wide variety of genetic pathways can be affected. The genes more frequently mutated are APC, KRAS, TP53, SMAD4 and PIK3CA (145). These mutations impair important signaling and regulatory pathways involved in proliferation, differentiation, and apoptosis (145). Besides, the mutations in KRAS indicate poor prognosis and predict resistance to anti-EGFR therapies (146).

CRC is a disease characterized by the progression of benign adenomas to malignant adenocarcinomas through a series of genetic and epigenetic alterations (147). There are two pathways that explain this transition from a benign adenoma to a malignant adenocarcinoma.

The first pathway is the one of sporadic CRC and includes a mutation in APC as an early event, followed by the accumulation of additional genetic alterations that activate oncogenes and inhibit tumor suppressor genes. Importantly, here the mutation of TP53 is a late event (147). This pathway is the most common. The second pathway is the one of colitis-associated CRC, which is characterized by chronic inflammation in the colon due to conditions such as ulcerative colitis or Chron's disease. In this pathway, the mutation of TP53 is an early event that occurs in the setting of chronic inflammation, leading to the development of dysplastic lesions that can mutate APC in a late event that drives the progression to adenocarcinoma (147). Noteworthy, both pathways occur in an inflammatory context but there are important differences between them. Sporadic CRC pathway occurs in a low-grade inflammation microenvironment enriched in IL-17 (148), and IL-23 signaling (149), whereas the colitis-associated CRC pathway is characterized by a high-grade inflammation involving IL-6 and TNF-α signaling (150).

Regarding the type of genetic alterations in the tumor, CRC can be classified into tumors with microsatellite instability and tumors with chromosomal instability and then further subclassified into 4 molecular subtypes (CMS1-4) (151). Tumors with microsatellite instability belong to CMS1 and are characterized by defects in DNA mismatch repair mechanisms, including mutations in genes such as MLH1, MSH2, MSH6, AND PMS2. This leads to the accumulation of mutations in microsatellite regions throughout the genome (152). Besides, tumors with microsatellite instability typically exhibit alterations in methylation patterns, such as hypermethylation of CpG islands and subsequent gene silencing (151). The high mutation burden of these types of tumors generates a high neoantigen load. Therefore, it is easier for the adaptive immune system to recognize and target these tumor cells (151,152). Consequently, tumors with microsatellite instability tend to have a high infiltration of Th1 and CD8+ T lymphocytes (151,152). Moreover, tumor cells in this context evade immune surveillance impairing the T lymphocyte response. This can be achieved through the upregulation of immune checkpoint molecules such as PD-L1 or CTLA-4, which bind to their receptors on T cells and suppress their activity (153).

Tumors with chromosomal instability, on the other hand, are characterized by large-scale genomic rearrangements and alterations in chromosomal structure (154). These alterations can lead to the loss or amplification of specific genes involved in tumor development and progression (154). Nevertheless, these tumors are much more heterogeneous and include CMS2-4 molecular subtypes (151). The CMS2 subtype is characterized by the activation of the WNT signaling pathway, which plays a crucial role in cell proliferation and differentiation (155). Besides, this subtype also exhibits high EGFR and MYC expression and amplification (155). CMS3 tumors have alterations in metabolic pathways such as the upregulation of fatty acid metabolism and downregulation of oxidative phosphorylation (156). Additionally, this subtype shows an increased epithelial-to-mesenchymal transition due to dysregulated TGF-β and NF-κB signaling pathways (156). Finally, CMS4 tumors are characterized by stroma and stromal cell infiltration, indicating a strong desmoplastic reaction (157). This subtype displays more aggressive behavior and is associated with a worse prognosis compared to the other subtypes (158).

The role of tumor microenvironment in colorectal cancer

CRC tumors are made of a complex network of different cell types, extracellular matrix components and signaling molecules that interact in a dynamic tumor microenvironment (TME). CRC includes neoplastic cells of epithelial origin organized in a hierarchical structure in which cells with different degrees of differentiation, phenotypes, and clonogenic potential coexist (159). Moreover, endothelial cells, fibroblasts, immune cells, and other stromal cells are also present in the TME (160). Importantly, most of the interactions in the TME are bidirectional. Therefore, non-neoplastic cells can modulate the behavior and characteristics of tumor cells, affecting their growth, invasion, and response to treatment (161) while neoplastic cells can influence extracellular matrix remodeling, angiogenesis, and immune phenotype (162). This crosstalk has a significant impact on tumor progression, metastasis, and response to therapy (163).

Chronic inflammation in the TME is a hallmark of cancer (160). This is particularly relevant in CRC since patients with conditions such as inflammatory bowel disease have a higher risk of developing CRC (147). When the colon is under stress it triggers a low-grade inflammatory response to adapt to the new condition. This low-grade inflammatory response is often referred to as para-inflammation, as it involves the moderate recruitment of immune cells and the release of cytokines that promote tissue repair and homeostasis (8). However, if the condition that is triggering this para-inflammation persists or becomes more severe it can progress to chronic inflammation and profoundly alter the microenvironment (8). This chronic para-inflammation becomes particularly relevant if epithelial cells undergo genetic mutations. The purpose of this chronic inflammation is to remove damaged cells, repair the tissue, and restore homeostasis in the affected area (164). This inflammatory microenvironment triggers the senescence inflammatory response (SIR) in epithelial cells (165). The SIR is a complex process involving the activation of various signaling pathways in which p53 plays a crucial role. If p53 is working properly, it can induce cell cycle arrest in damaged cells, allowing for their removal from the tissue as well as the controlled proliferation of surrounding healthy cells to replace the damaged ones (166). If p53 is not working, it can lead to the accumulation of damaged cells, uncontrolled proliferation, and subsequent tumor development (166). Importantly, senescent cells that are not removed acquire a secretory phenotype known as the senescent-associated secretory phenotype (SASP). The SASP is characterized by the secretion of various pro-inflammatory cytokines, growth factors, and matrix-degrading enzymes. This contributes to the pro-inflammatory microenvironment, generates stress in the surrounding cells, and promotes tumor development and progression (165). In short, acute inflammatory processes contribute to the efficient removal of damaged cells and reduce the chances of tumor development, while classical chronic inflammation and para-inflammation contribute to the survival of senescent cells and increase the risk of tumor development.

This phenomenon is important to understand treatment resistance. Chemotherapy works by inducing DNA damage. This is lethal in rapidly dividing cells like tumor cells, but it also causes stress and DNA damage-induced cellular senescence in normal cells, leading to the secretion of SASP factors and the promotion of inflammation, which in turn can contribute to resistance to

these therapies (167). Besides, not all tumor cells are dividing, and some may exist in a quiescent state, which makes them less susceptible to the cytotoxic effects of chemotherapy (168). Therefore, the reorganization of the microenvironment after the therapy can trigger the selection of clones, resulting in the emergence of tumor cells that have acquired resistance to treatment, and the activation of quiescent tumor cells that were previously unaffected by the treatment (169). The SASP produced by stressed and senescent cells after chemotherapy contributes to the development of a pro-inflammatory microenvironment promoting tumor cell survival, proliferation, and resistance to therapy (170). However, the TME undergoes a profound remodeling after the treatment, which includes changes in the composition of immune cells, cytokine levels and extracellular matrix components, all of which can influence tumor growth and response to therapy (171).

From the immunological perspective, tumors with chromosomal instability exhibit a reduced neoantigen burden compared to tumors with microsatellite instability, which results in a weaker activation of the adaptive immune system and a reduced infiltration of T lymphocytes into the TME (172). However, there are important differences between CMS2-4 subtypes in terms of their immunological characteristics. CMS2 tumors have a significantly lower infiltration of Th1 and CD8+ T lymphocytes than CMS1 tumors, but there is still some infiltration of these cells into the TME (173,174). Moreover, the dominant immune cell populations in the TME of CMS2 tumors are resting NK cells and naive CD4+ T lymphocytes (173,174). This TME also includes the upregulation of CXCL9, CXCL10, CXCL16, and IFN-γ, which contribute to the recruitment of NK and T lymphocytes and the upregulation of MHC class I in the TME (175). Furthermore, these tumors also upregulate immune checkpoint molecules to evade immune surveillance (176). Importantly, both CMS1 and CMS2 are immune-activated TMEs, the difference is the degree of activation. In addition to the higher infiltration of Th1 and CD8+ T lymphocytes, CMS1 tumors also exhibit a higher infiltration of T follicular helper cells and a higher activation of γδ T lymphocytes and dendritic cells (177). Besides, macrophages in CMS1 are more polarized toward pro-inflammatory phenotypes while in CMS2 they are more polarized toward anti-inflammatory phenotypes (178).

On the other hand, CMS3 and CMS4 tumors show an extremely reduced or absent infiltration of Th1 and CD8+ T lymphocytes, indicating a more immunosuppressive TME (173,174). CMS3 is considered an immune desert due to the almost absent immune infiltration. The very few immune cells present in the TME include macrophages with an anti-inflammatory phenotype and some neutrophils (177).

CMS4 tumors exhibit a high infiltration of anti-inflammatory macrophages, Tregs, and myeloid-derived suppressor cells (MDSCs) (173,178). These cells generate a non-productive inflammation that promotes tumor progression and immunosuppression mainly by the upregulation of TGF-β and VEGF (173,174). This is enhanced by the upregulation of immune checkpoint molecules, which further support immune evasion and yield a worse prognosis (179,180). Importantly, CMS4 is much more heterogeneous than the other subtypes and some authors have suggested that it should be further subdivided into additional subgroups based on the specific immune cell composition and molecular characteristics (181,182).

Noteworthy, neutrophils are present in all molecular subtypes and mast cells are only absent in CMS3. However, their specific role in the TME of each subtype is still not fully understood and requires further investigation (183). Finally, approximately 13% of all CRC tumors cannot be classified in any of the CMS groups (182), which indicates the presence of alternative molecular subtypes or unique immune microenvironments that have not yet been characterized.

In a nutshell, when it comes to TME, CMS1 tumors exhibit a high immune activation, CMS2 tumors a low immune activation, CMS3 tumors immune exclusion, and CMS4 tumors immunosuppression. Nevertheless, immune evasion occurs in all of them and is essential for tumor progression.

This highlights the importance of understanding the TME and its interactions to improve cancer treatment strategies and overcome therapy resistance. Accordingly, immunotherapies have been developed to target the TME and complement chemotherapy treatments. The discovery of immune checkpoints offered a new strategy for cancer treatment by blocking the inhibitory signals that suppress the immune system's ability to recognize and eliminate tumor cells (184). In CRC, these immunotherapies focused mainly on blocking the PD-1/PD-L1 and CTLA-4 pathways, which are critical regulators of T cell responses in the TME (185). Unfortunately, only patients with CRC tumors that display microsatellite instability respond to immunotherapy (186). This accounts only for 5-15% of all CRC patients (187), highlighting the complexity and heterogeneity of the TME and the need for further research and understanding of the interactions between tumor cells, immune cells, and the TME.

The importance of tumor microenvironment in clinical decisions. State of the art and perspectives

Traditionally, the staging of CRC has been performed with the use of the American Joint Committee on Cancer TNM staging system, which considers the tumor size and invasion, lymph node involvement, and presence of distant metastases (188). Stage 0 is known as carcinoma in situ, where the tumor is confined to the innermost layer of the colon or rectum. Stage I CRC is characterized by invasion of the tumor into the submucosa, while stage II CRC indicates penetration through the muscularis propria but without involvement of nearby lymph nodes. Stage III CRC is defined as the invasion of the tumor into nearby lymph nodes, and stage IV CRC implies distant metastasis to other organs (189). Noteworthy, these stages are further subclassified (from A to C) based on other factors such as tumor grade, histological type, and the presence of certain genetic mutations (189). This system may seem to be straightforward and universally applicable, but it has important limitations. For example, patients in stages IIB and IIC have worse outcomes compared to those in stage IIIA, which is not consistent with the expected progression of disease based on tumor size and lymph node involvement alone (189). This highlights the need for a more comprehensive and accurate prognostic scoring system.

The TNM system only uses information from anatomical features from tumor cells to stage colorectal cancer, but it fails to capture the complex interactions and dynamics of the TME that contribute to disease progression. Besides, its prognostic value is limited and fails to predict the response to treatment (190). Therefore, a new immunohistochemical system called immunoscore has been developed to evaluate the immune landscape within tumors, taking into account the type, density, and location of immune cells (191). Currently, the immunoscore algorithm evaluates the infiltration of total T lymphocytes and CD8+ T lymphocytes in the tumor core and invasive margin and gives a score from 0 to 4 in each location (192). Interestingly, accumulating evidence suggests that the immunoscore has shown superior and independent prognostic value than TNM and helps to predict the response to adjuvant chemotherapy (191,193). Moreover, the clinical guidance for the management of patients with CRC from the European Society of Medical Oncology (ESMO) includes the assessment of immunoscore as a recommended prognostic marker and guide for treatment decisions (194).

Despite the huge leap forward of immunoscore in the management of patients with CRC it still has some weaknesses. One of the main drawbacks is that it currently focuses mainly on the evaluation of CD8+ T lymphocytes, neglecting the contributions of other immune cell types in the TME. Besides, given the current knowledge about the molecular subtypes of CRC, the sole measurement of CD8 T lymphocytes may not be appropriate in all the subtypes. In the case of CMS1 and CMS2, since they are characterized by higher immune infiltrates of CD8+ T lymphocytes and their blockage through immune checkpoints is one of the main strategies for immune evasion, the immunoscore may provide valuable information about the stage of the tumor. In CMS3, since it is an immune desert, the immunoscore may not be as informative in terms of predicting the prognosis and the response to treatment. Finally, in CMS4 there is not an easy answer due to the complexity of the interactions in its TME and its heterogeneity. Some CMS4 tumors can exhibit certain degree of CD8+ T lymphocyte infiltration (178). However, this is one of the main sources of heterogeneity inside the CMS4 subtype (195). Besides, in the CMS4 subtype the recruitment and activation of Tregs and MDSCs are much more relevant than in the other subtypes (183,195) and it may be a better target for the immunoscore in this particular subtype of CRC. Importantly, the studies about immunoscore usually do not specify the molecular subtypes of colorectal cancer that were included in their analyses. CMS1 and CMS2, in which current immunoscore should have a very high predictive value, represent respectively 14 and 37% of all CRC cases, while CMS3, in which current immunoscore should not have a high predictive value, represents about 13% of all cases (196). Moreover, CMS4 and tumors that do not fall into any of these subtypes, in which is unclear whether CD8+ lymphocyte infiltration is a good indicator of tumor progression, represent respectively about 23 and 13% (196). Therefore, in a random selection of patients with CRC, it may be possible that the good predictive results of patients with CMS1 and CMS2 will compensate for the results of patients with CMS3, leading to an overall favorable predictive value of the immunoscore. In any case, the use of different immunoscores in different subtypes of CRC may be more useful for tailoring treatment strategies and predicting prognosis accurately.

This problem will have a difficult solution with the current immunoscore set up, since in immunohistochemistry you can only test one marker at a time (197), making it challenging to

comprehensively assess all immune cell populations in the TME. It would be possible to adapt the technology to multiplex immunohistochemistry or immunofluorescence, which would allow the simultaneous detection of a few markers (198). Nevertheless, that would be still insufficient. The best option for keeping the spatial information and evaluate multiple immune cell types simultaneously would be the use of advanced technologies such as spatial transcriptomics or spatial proteomics (199). The problem with these cutting-edge technologies is that they are currently limited in their availability, very expensive, and require further validation and standardization before they can be widely adopted in clinical practice. Besides, the data generated by these advanced technologies can be complex and may require sophisticated bioinformatic analysis to extract meaningful information from them. Such could be a problem for small and medium-sized medical facilities that may not have the resources or expertise to effectively utilize these technologies.

In this challenging scenario, flow cytometry emerges as a very interesting approach. Flow cytometry is a powerful tool routinely used in clinical practice that allows the simultaneous detection of several markers and their quantification in individual cells (200). It is frequently used in hematology for the diagnosis, treatment decision, and monitoring of hematologic malignancies (201). However, when applied to solid samples previously digested, the partially undigested extracellular matrix can interfere with the analysis (202). Thus, in clinical practice, flow cytometry is currently limited to peripheral blood and bone marrow samples (201). The advantages of developing an immunoscore version with flow cytometry include the ability to check for multiple immune cell markers simultaneously, allowing for a comprehensive assessment of the immune cell populations in the TME. Additionally, flow cytometry offers the possibility of providing quantitative data on marker expression levels, allowing for a more precise characterization of immune cell phenotypes within the TME (203). This is very relevant since the production of cytokines and chemokines can be measured along with surface markers (203), providing additional insights into the functional state and immune response within the tumor. Noteworthy, the abundance of certain cytokines and chemokines has prognostic value in CRC (191) and in other types of cancer has been seen that it predicts treatment response (204). Besides, since most hospitals already have flow cytometry instruments and expertise in place, it would make the implementation of immune phenotyping via flow cytometry a more feasible and cost-effective option. Nevertheless, unlike immunohistochemistry and the advanced technologies mentioned earlier, flow cytometry does not provide spatial information, which may limit its applicability for the analysis of solid tumors (205). However, the fact that the number of cells in the cell suspension employed for flow cytometry is much higher than what can be obtained from tissue sections (203) brings two important advantages. Firstly, samples from multiple regions of the tumor can be combined, providing a more representative and comprehensive analysis of the heterogeneity of the TME and potentially overcoming the limitations of the lack of spatial information. Secondly, it allows the identification and analysis of rare cell populations that may be present in low frequencies within the TME.

In summary, the use of flow cytometry in immunoscore development offers several advantages, including the ability to assess multiple immune cell markers simultaneously, to provide quantitative data on marker expression levels, and an easy translation into clinical practice.

Hypothesis

- The tumor microenvironment (TME) induces changes in the dynamics of innate immune cells to promote tumor progression and avoid immune surveillance. This will include changes in the immunophenotype mainly in mast cells, since they are critical players in the modification of the extracellular matrix.

Objectives

- To develop a protocol that allows for the obtention of high-quality cell suspensions from mucosa and CRC samples.
- To evaluate the changes in population dynamics in T lymphocytes, macrophages, neutrophils, eosinophils, plasmacytoid dendritic cells, conventional dendritic cells, and mast cells between the mucosa of healthy controls, the unaffected mucosa of CRC patients, and CRC.
- To evaluate the surface abundance of CD45 on innate immune cells.
- To evaluate the immunophenotypical changes in mast cells between the mucosa of healthy controls, the unaffected mucosa of CRC patients, and CRC.

Materials and Methods

Cohort

This study includes 54 untreated patients diagnosed with CRC and 20 healthy controls that underwent routine tests. Patients with synchronic tumors, previous oncological diseases, Lynch syndrome, and systemic autoimmune diseases were excluded from the cohort. All patients accepted to participate in the study and provided their written informed consent.

Sample Processing

For each patient with CRC, two biopsies were collected, one from the tumor and one from the nearby unaffected mucosa (3 cm away from the tumor margin). Besides, healthy controls provided one sample from normal mucosa. All the samples were collected in the Gastroenterology Unit of San Cecilio University Hospital following approved protocols. The biopsies were conserved in PBS and ice right after their extraction. After that, they were digested in RPMI 1640 medium supplemented with 2 mg/ml collagenase type I (Sigma-Aldrich), hyaluronidase (Sigma-Aldrich), chondroitinase (Sigma-Aldrich), heparinase I-III (Sigma-Aldrich) and 5 mM $CaCl_2$ in a final volume of 1 ml, at 37°C during 2h. Subsequently, the samples were poured through a 100μm filter, and centrifuged for 10 minutes at 900 x g. Finally, the pellet,

that includes the stromal vascular fraction, was resuspended in 500µl of antibody staining buffer (PBS, 2% fetal bovine serum, 0.09% albumin, and 0.05% sodium azide) and mixed with 50µl an internal standard (BD Truecount Absolute Counting Tubes) following manufacturer instructions.

Antibody Staining and Flow Cytometry

The stromal vascular fraction was stained with 2ml of appropriate controls or fluorophore-labaled antibodies in Eppendorf tubes at room temperature for 20 minutes. Later, the cell suspension was fixed, and erythrocytes lysed with 1 ml of BD FACS Lysing Solution for 30 minutes. Then, the samples were centrifuged 10 minutes at 3500 x g, and the pellets were resuspended in 500µl of PBS. After that, the samples were stored at 4°C until the next day. Flow cytometry was performed using a FACS ARIA III equipment, and data were acquired on a logarithmic scale. The internal standard was used to calculate the number of cells per mg of tissue and to normalize the fluorescence signal when addressing the surface abundance of the different proteins. Compensation beads and isotype controls were purchased from BD Biosciences. The complete list of antibodies employed in this study is detailed in the annex. The different cell subpopulations were identified as follows: $CD4^+$ T lymphocytes ($CD45^+$ $CD3^+$ $CD4^+$ $CD8^-$), CD8+ T lymphocytes ($CD45^+$ $CD3^+$ $CD4^-$ $CD8^+$), macrophages ($CD45^+$ $CD66b^-$ $CD117^-$ $CD14^{+/-}$), neutrophils ($CD45^+$ $CD66b^+$ $CD117^-$ $CD14^-$ $CD16^+$), eosinophils ($CD45^+$ $CD66b^+$ $CD117^-$ $CD14^-$ $CD16^-$), plasmacytoid dendritic cells (Lin^- $CD45^+$ $CD11c^-$ $CD123^+$), conventional dendritic cells (Lin^- $CD45^+$ $CD11c^+$ $CD123^-$), mast cells ($CD45^+$ $CD117^+$ $FceRI^+$)

Single cell RNA seq (scRNA seq)

The raw count file was downloaded from the NCBI Gene Expression Omnibus with the accession code GSE200997. The analysis was performed with R software and Seurat package. For quality purposes, genes expressed in less than 3 cells as well as cells expressing less than 200 genes, more than 5000 genes, or with an enrichment of more than 30% in mitochondrial were removed from the analysis. After that the data was log normalized, variable genes were selected through variance stabilizing transformation, and data was scalated. Then, a PCA was performed followed by an UMAP with the top 50 principal components. Later, the clustering was performed using the Lovain algorithm. Each cell type was identified with the following markers: neutrophils (CSF3R, S100A8, S100A9), plasmacytoid dendritic cells (LILRA4, LRRC26), conventional dendritic cells (CD1A, CD1C), and mast cells (TPSAB1, TPSB2).

Statistical Analysis

The differences between healthy controls, unaffected mucosa, and CRC were analyzed with the Kruskal Wallis test followed by a Wilcoxon pairwise test. All the tests were conducted with R software and p-values below 0.05 were considered statistically significant.

Results

Flow cytometry

Figure 39 highlights the importance of carbohydrate digestion to obtain high quality cell suspensions. Importantly, all those undigested events increase the amount of time that the sample needs to be analyzed in the flow cytometer. Besides, due to the carbohydrate nature of these events they can aggregate between them due to labile interactions. Sometimes they can aggregate after passing the filter of the sample line of the flow cytometer generating a clog that blocks the flow cytometer and forces to stop the experiment to remove it. Therefore, the addition of chondroitinase, heparinase I-III, and hyaluronidase in the digestion cocktail is critical for the success of the experiment. Figure 40-Figure 43 show the gating strategy employed to identify the different immune cell populations.

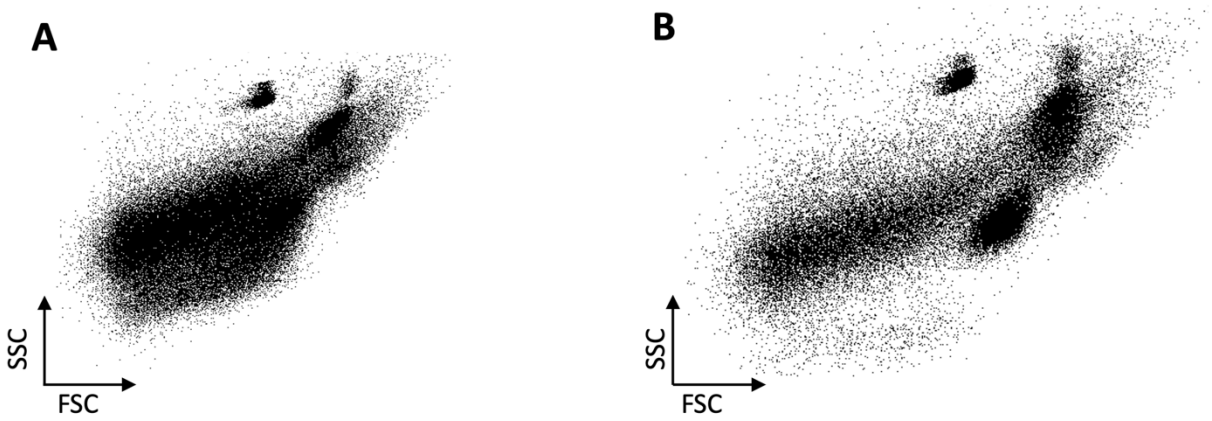

Figure 39. Effect of carbohydrate digestion in the quality of the cell suspension. A) Cell suspension from a digestion with collagenase I. B) Cell suspension from a digestion with collagenase I, chondroitinase, heparinase I-III and hyaluronidase

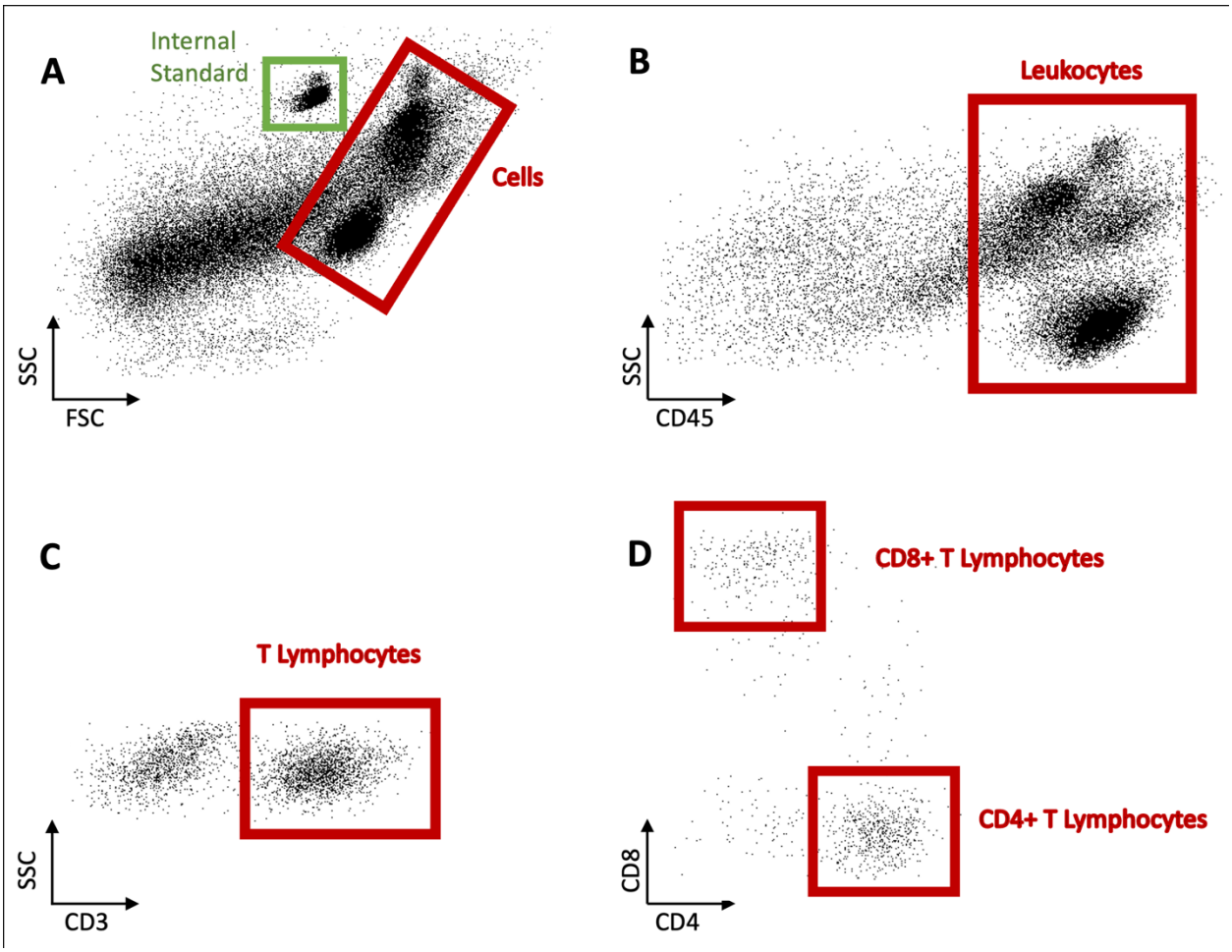

Figure 40. Gating strategy for the identification of T lymphocyte subpopulations. A) Identification of cells and the internal standard. B) Identification of leukocytes. C) Identification of T lymphocytes. D) Identification of CD4+ and CD8+ T lymphocytes.

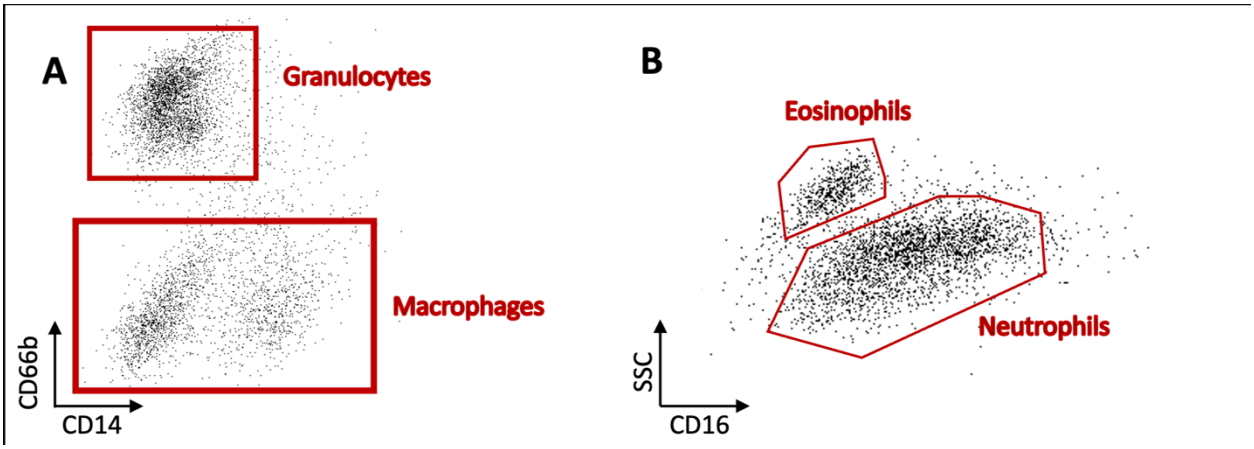

Figure 41. Gating strategy for the identification of macrophages and granulocytes. A) Identification of macrophages and granulocytes. B) Subclassification of granulocytes into neutrophils and eosinophils.

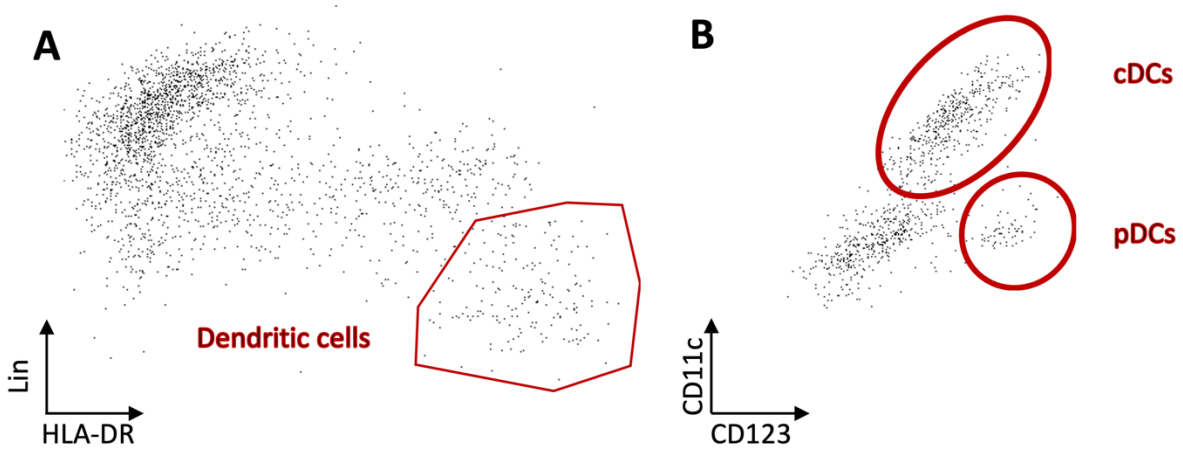

Figure 42. Gating strategy for the identification of dendritic cell subpopulations. A) Identification of dendritic cells. B) Subclassification of dendritic cells into plasmacytoid (pDCs) and conventional (cDCs). Lin (CD3, CD19, CD56, CD66b, CD14, CD117)

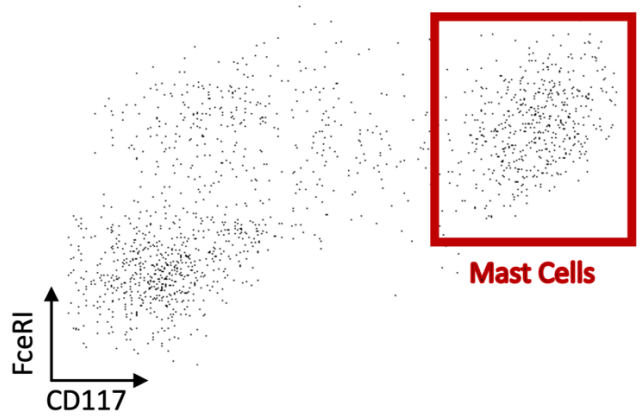

Figure 43. Gating strategy for the identification of mast cells

The number of macrophages and T lymphocytes increases in colorectal cancer

Macrophages and T lymphocytes have been extensively studied in the tumor microenvironment (TME). The number of macrophages in the tumor microenvironment increases due to the recruitment of peripheral monocytes. After that, they acquire a tumor-promoting phenotype that facilitates tumor growth and immune evasion (206). Figure 44 shows that the number of macrophages is higher in tumors than in the unaffected mucosa of the same patients and in the mucosa of healthy controls. Thus, this result agrees with previous reports.

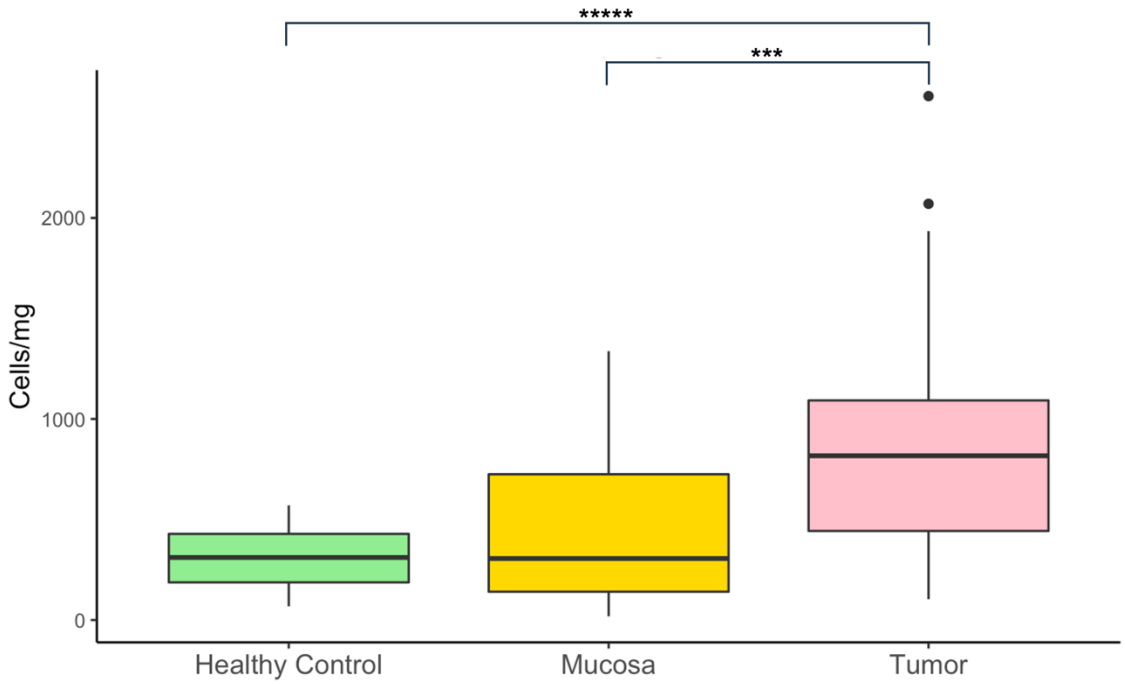

Figure 44. Number of macrophages per mg of tissue in the flow cytometry cohort. Tumor (n=42), unaffected mucosa (n=42), healthy control (n=20).

T lymphocytes are critical cells in the TME. They are recruited from peripheral blood and can play a pro or anti-tumoral role. Although some $CD4^+$ T lymphocytes in the TME can have a Th1 phenotype and play an anti-tumoral role, most of them have a Th2 or Treg phenotype that promotes tumor growth and immunosuppression (207). Therefore, the number of $CD4^+$ T lymphocytes in the TME increases as the tumor grows.

$CD8^+$ T lymphocytes are effector cells that destroy tumor cells efficiently. A high number of $CD8^+$ T lymphocytes in the tumor is a good prognostic factor (208). However, as the tumor grows, it impairs the function of $CD8^+$ T lymphocytes by several mechanisms: downregulating HLA-I, upregulating immune-checkpoints, or recruiting immunosuppressive cells (209). Therefore, the number of $CD8^+$ T cells increases but not as much as the $CD4^+$ cells due to this inhibition (210). Figure 45 and Figure 46 show that the number of both $CD4^+$ and $CD8^+$ lymphocytes rises in the tumor, but this increase is more prominent in the $CD4^+$ population. Thus, the present study agrees with the current bibliography.

The results obtained in the analysis of macrophages and T lymphocytes in the gut mucosa confirmed previous observations (206–208,210). Then, although they cannot be considered as novel results, these have validated the way by which the samples have been processed. Thus, allowing the achievement of reliable results.

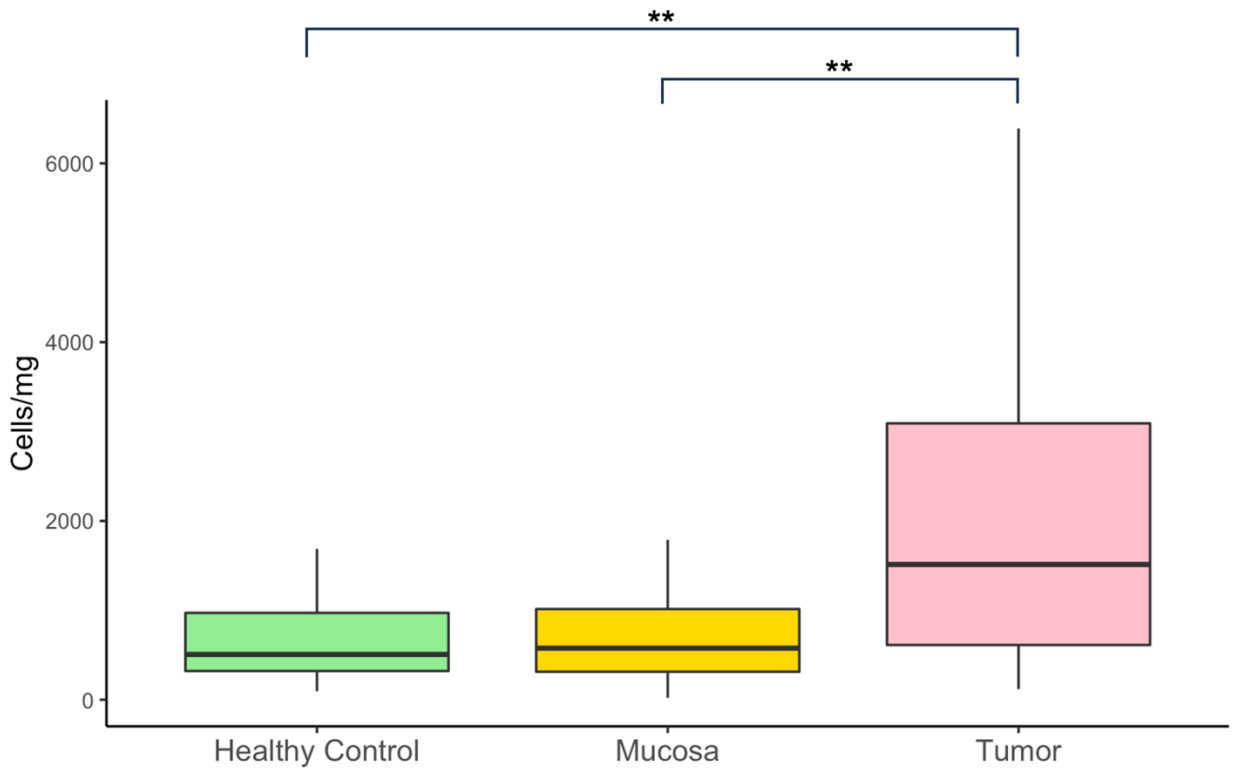

Figure 45. Number of CD4⁺ T lymphocytes per mg of tissue in the flow cytometry cohort. Tumor (n=31), unaffected mucosa (n=31), healthy control (n=14).

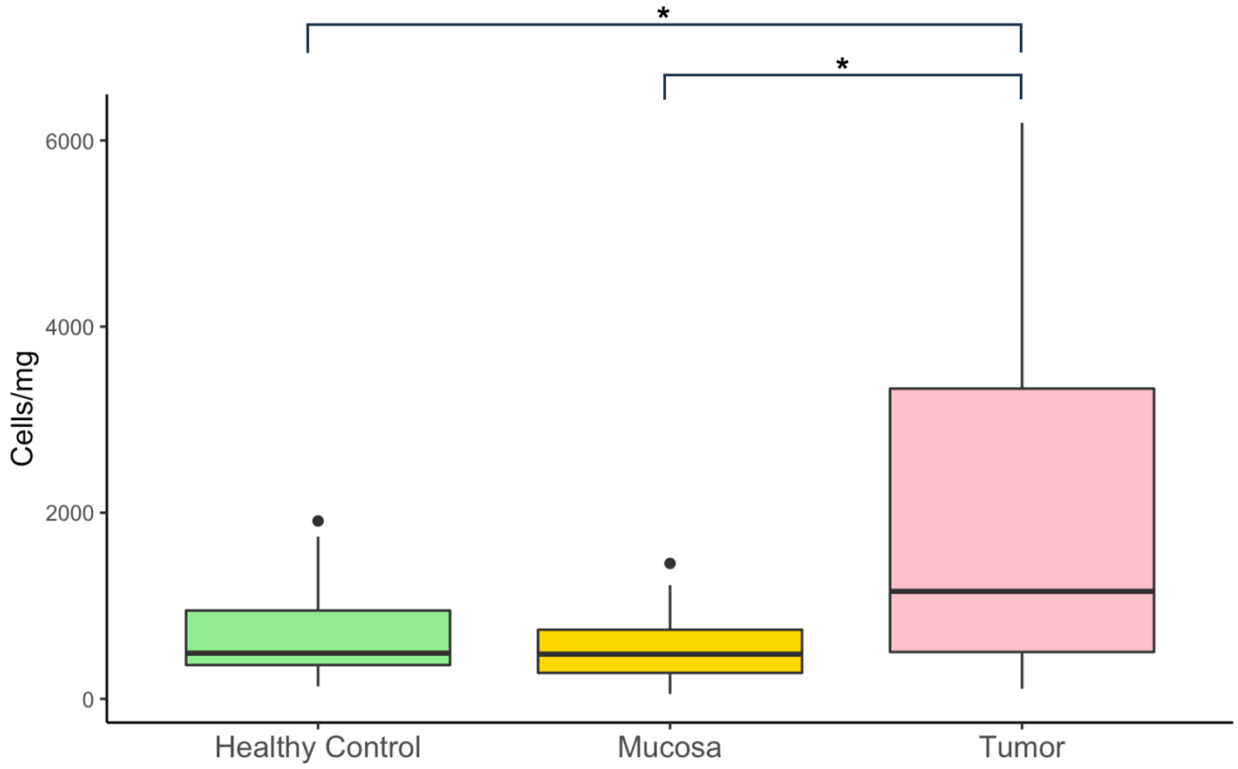

Figure 46. Number of CD8⁺ T lymphocytes per mg of tissue in the flow cytometry cohort. Tumor (n=31), unaffected mucosa (n=31), healthy control (n=14).

The number of neutrophils increases while their surface abundance of CD45 decreases in colorectal cancer

Neutrophils are the most abundant cell type in peripheral blood and its role in CRC is strongly dependent on the interactions in the TME. Like macrophages they can have an anti-tumoral (N1) or pro-tumoral (N2) phenotype. Several reports identified that the neutrophil to lymphocyte ratio increases in advanced CRC tumors and has prognostic value (211,212). However, some of these reports identified neutrophils as MPO positive cells, while some subpopulations of macrophages can also express MPO (213). The controversy increased after the discovery that a high number of neutrophils is a good prognostic factor for chemotherapy response (213). Besides, neutrophils and $CD8^+$ T lymphocytes usually colocalize in the TME and its physical interaction enhance the effector function of $CD8^+$ T lymphocytes (214). In the present study, CD66b was used for an accurate identification of neutrophils, and Figure 47 demonstrates that their number sharply rises in tumors compared to both unaffected mucosa and healthy controls (the axis is in logarithmic scale). Moreover, the number is higher in the unaffected mucosa of patients with CRC than in healthy patients which suggests that the tumor is causing an inflammatory stress in the nearby mucosa. Figure 48 shows that in the scRNA seq dataset the proportion of neutrophils is much higher in the tumor than in the mucosa, which validates the results obtained from the flow cytometry. Figure 49 illustrates the abundance of CD45 on the plasma membrane of neutrophils, which is significantly higher in healthy controls. CD45 is an important positive regulator for neutrophil effector functions (215). Therefore, this data suggests that this neutrophil effector function is lost in the tumor.

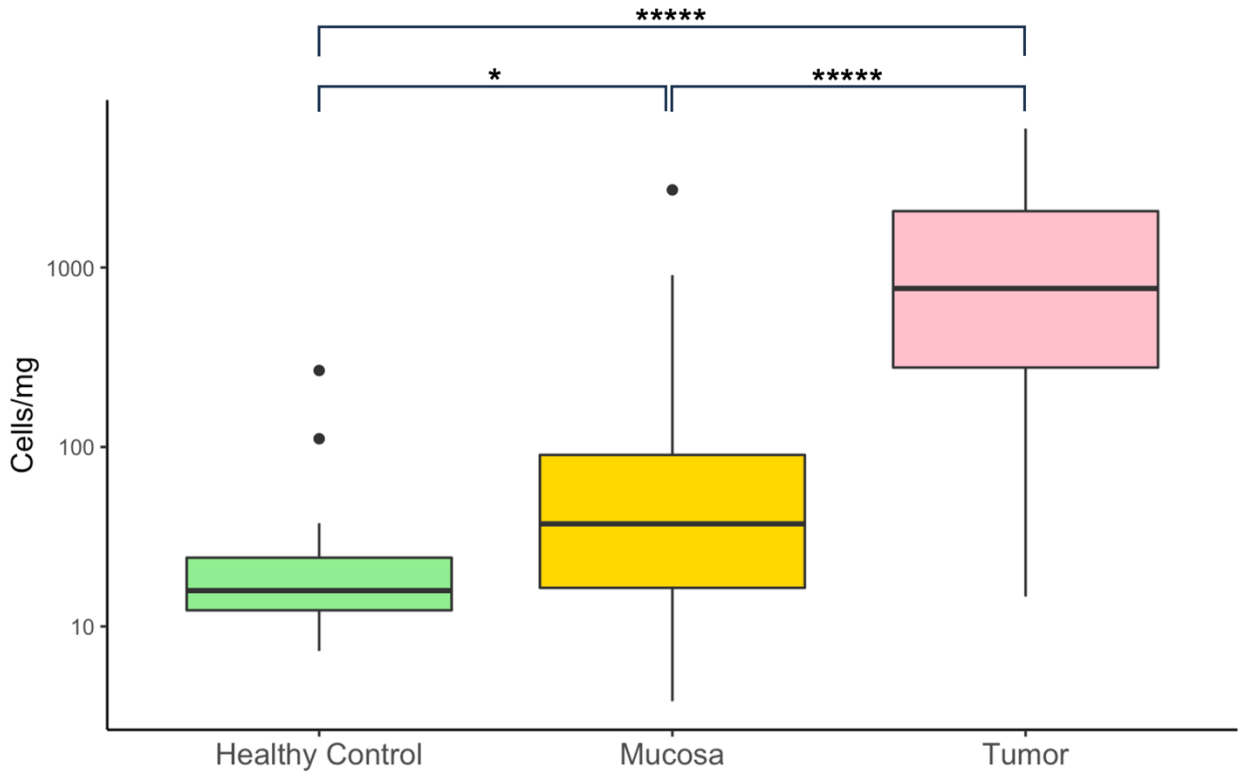

Figure 47. Number of neutrophils per mg of tissue in the flow cytometry cohort. Please notice that the y-axis is in logarithmic scale. Tumor (n=48), unaffected mucosa (n=48), healthy control (n=19).

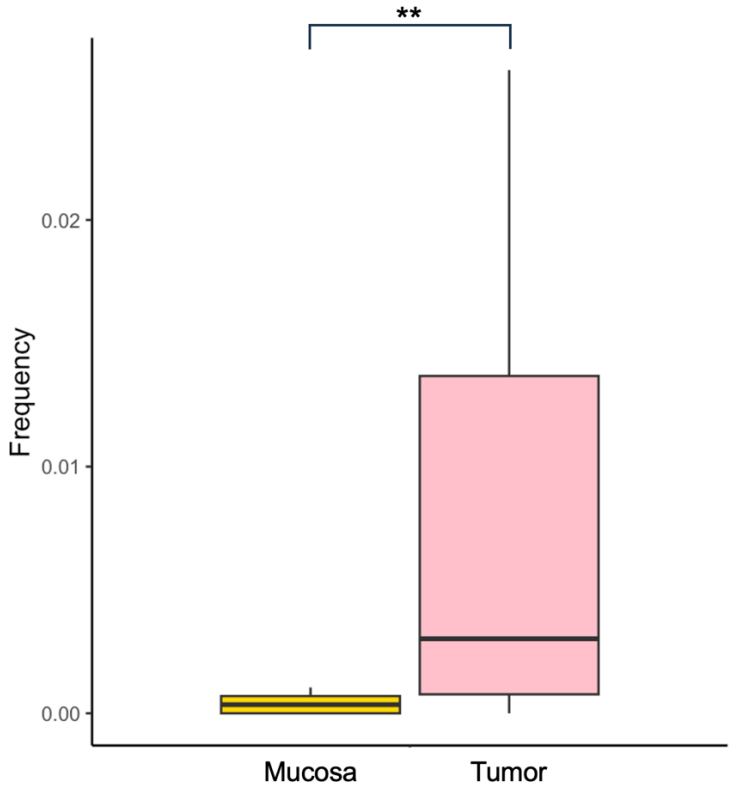

Figure 48. Relative abundance of neutrophils in the scRNAseq cohort. Tumor (n=15), unaffected mucosa (n=7).

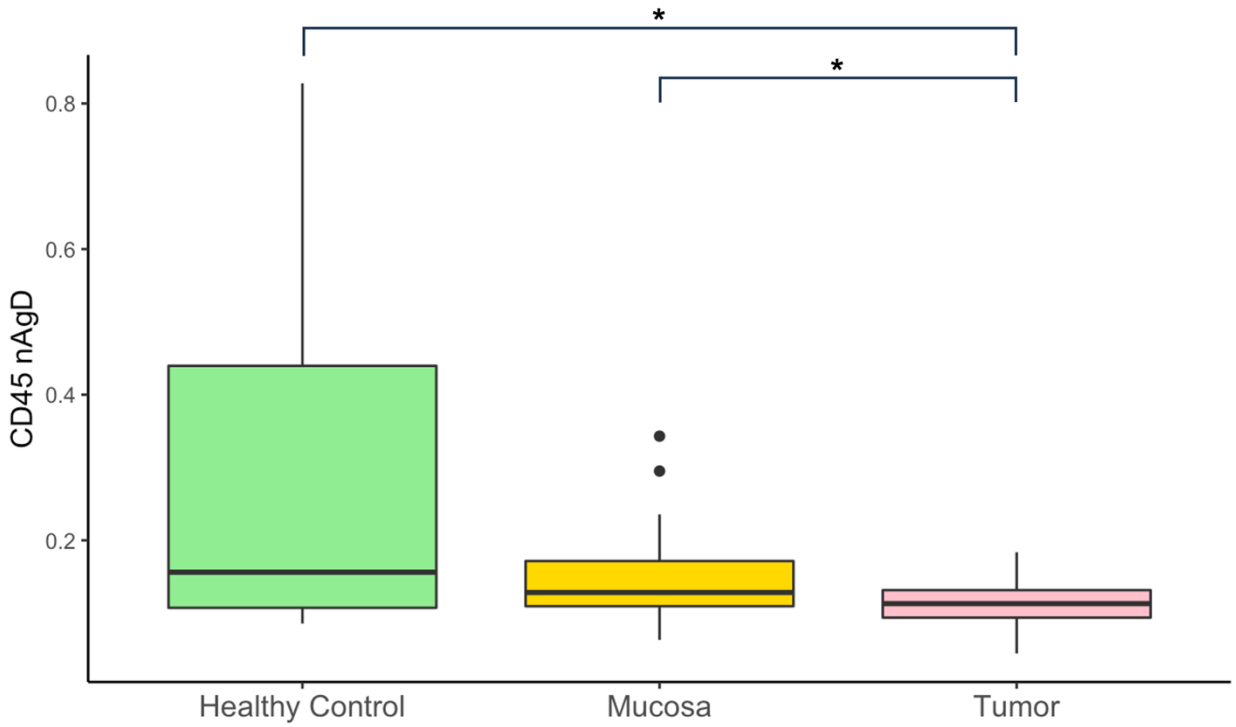

Figure 49. Normalized antigenic density (nAgD) of CD45 on the surface of neutrophils in the flow cytometry cohort. Tumor (n=48), unaffected mucosa (n=48), healthy control (n=19).

The number of eosinophils and their surface abundance of CD45 decrease in colorectal cancer

Eosinophils are pivotal cells in the immunosurveillance of the gastrointestinal tract mucosa (216). Noteworthy, they play an important anti-tumoral role. They act as effector cells releasing cytotoxic compounds and extracellular traps that kill tumor cells. Besides, they can also modulate other immune cells in the TME releasing IL-1, TNF-α, and IFN-β (217,218). Figure 50 shows a massive reduction in the number of eosinophils of both tumor and unaffected mucosa compared to the healthy control. Since eosinophils play an antitumoral role, it makes sense that the tumor finds ways to impair its recruitment and survival. Interestingly, this exclusion of eosinophils occurs also in the unaffected mucosa of patients with CRC. Unfortunately, it was impossible to identify an eosinophil cluster in the scRNA seq dataset. This is probably due to a combination of factors including the low initial number of eosinophils, small sample size, and the loss of eosinophils during the processing of the sample for the sequencing (eosinophils are very fragile).

Importantly, Figure 51 displays the amount of CD45 in the surface of eosinophils, which shows a massive decrease in the tumor and unaffected mucosa compared to the healthy controls. The role of CD45 in eosinophils is very poorly understood. However, it seems to play a role in its

activation by promoting the upregulation of CD11b and the shedding of CD62L (219). Nevertheless, the specific role of CD45 in tumor eosinophils needs to be further studied.

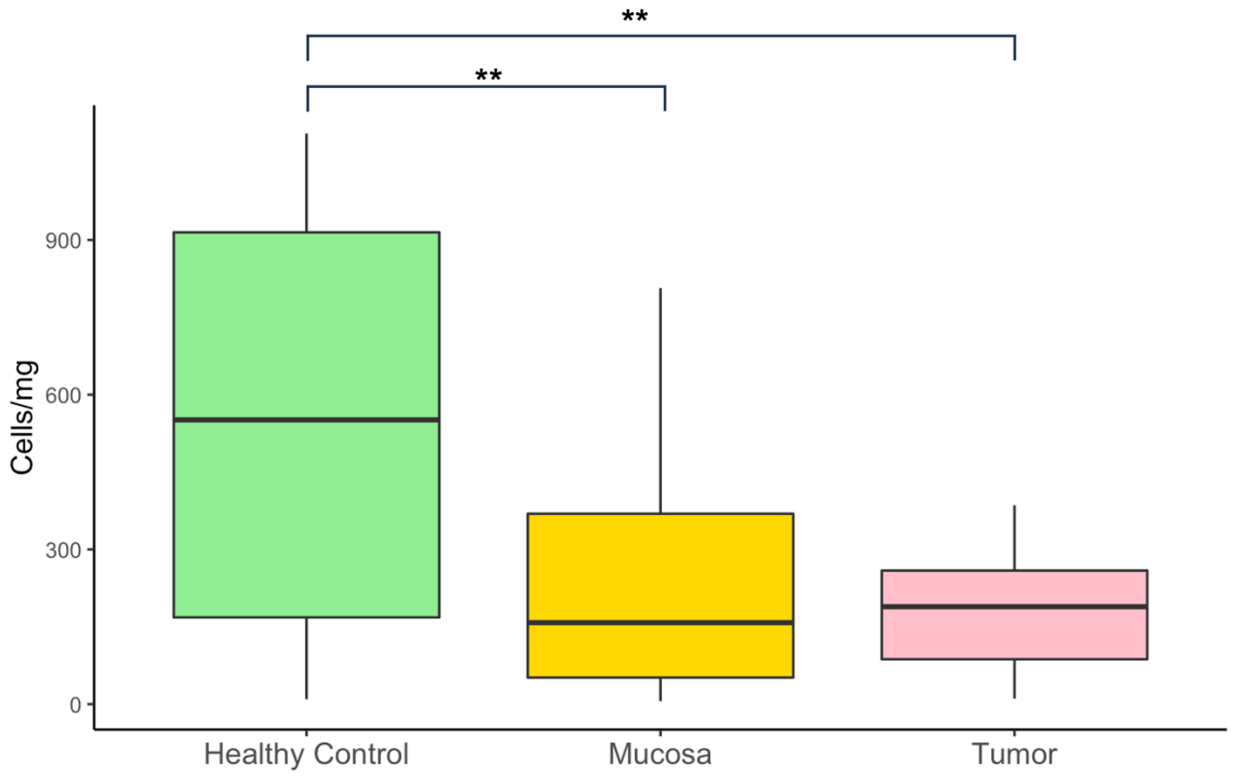

Figure 50. Number of eosinophils per mg of tissue in the flow cytometry cohort. Tumor (n=48), unaffected mucosa (n=48), healthy control (n=19).

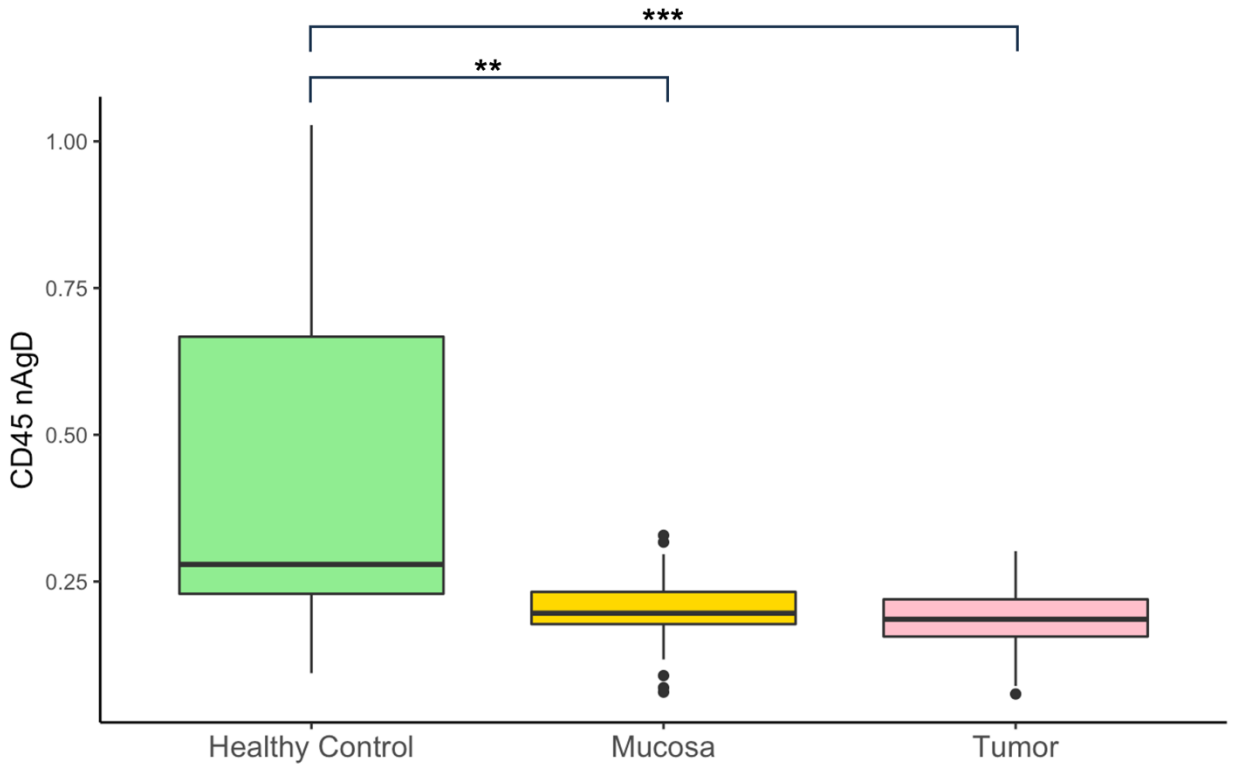

Figure 51. Normalized antigenic density (nAgD) of CD45 on the surface of eosinophils in the flow cytometry cohort. Tumor (n=48), unaffected mucosa (n=48), healthy control (n=19).

The number of plasmacytoid dendritic cells increases while their surface abundance of CD45 decreases in colorectal cancer

Plasmacytoid dendritic cells (pDCs) are widely known to produce type I IFNs in antiviral responses. However, in the TME, their differentiation to pro-inflammatory and antitumoral phenotypes is impaired. Then, they differentiate to tumor-supporting phenotypes. They release IL-10 and TGF-β that contribute to Treg differentiation and immune suppression (220). Figure 52 demonstrate that the number of pDCs is higher in the tumor which is coherent with their pro-tumoral role previously described. Besides, it seems to be a trend of higher number of pDCs in the unaffected mucosa than in the healthy control. This is probably not statistically significant because the number of samples is smaller than in other panels. Accordingly to Figure 53, the results of the scRNA seq agree with the results of the flow cytometry.

Noteworthy, Figure 54 highlights that pDCs have significantly more CD45 on their plasma membrane in healthy controls than in the tumor or the unaffected mucosa. Previous reports on dendritic cells showed that CD45 modulate TLR-signaling through the interaction with MyD88 and TRIF to enhance the production of pro-inflammatory cytokines (221). Therefore, since the tumor impairs the polarization towards pro-inflammatory phenotypes in pDCs (220) it seems reasonable to observe higher levels of CD45 in the healthy controls.

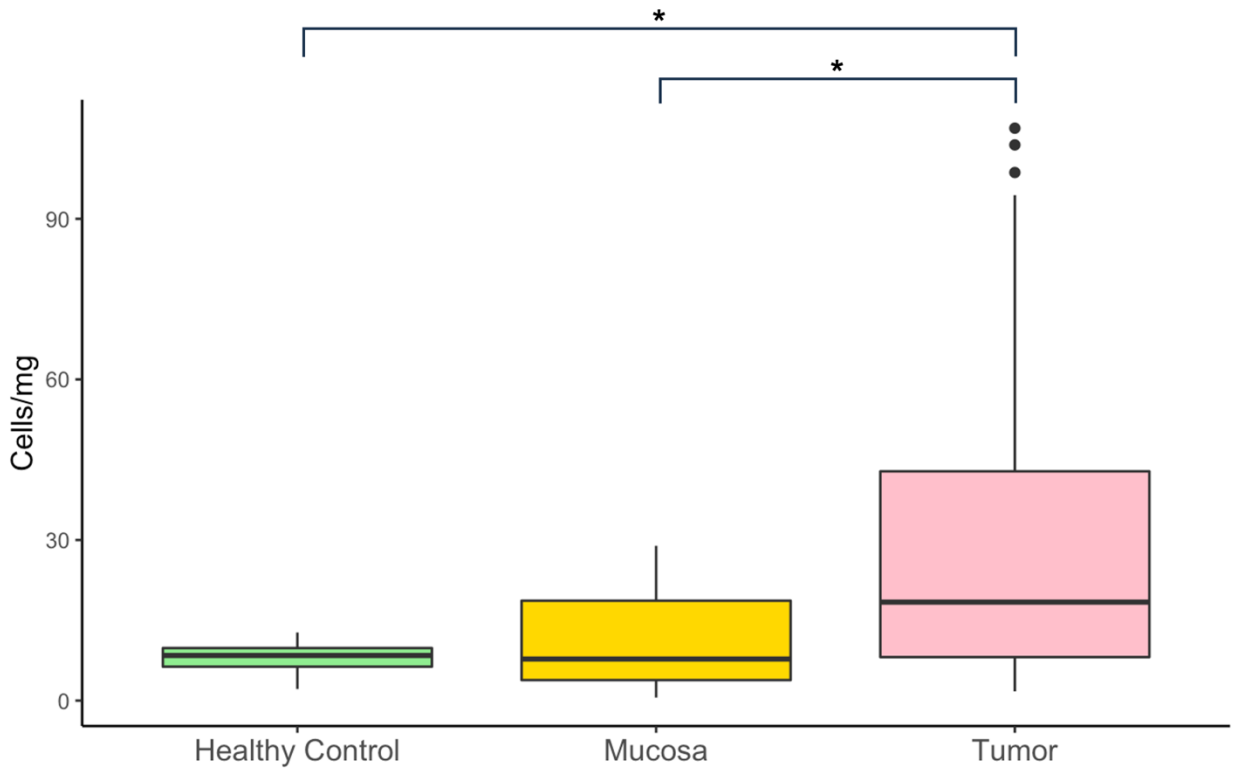

Figure 52. Number of plasmacytoid dendritic cells per mg of tissue in the flow cytometry cohort. Tumor (n=28), unaffected mucosa (n=28), healthy control (n=16).

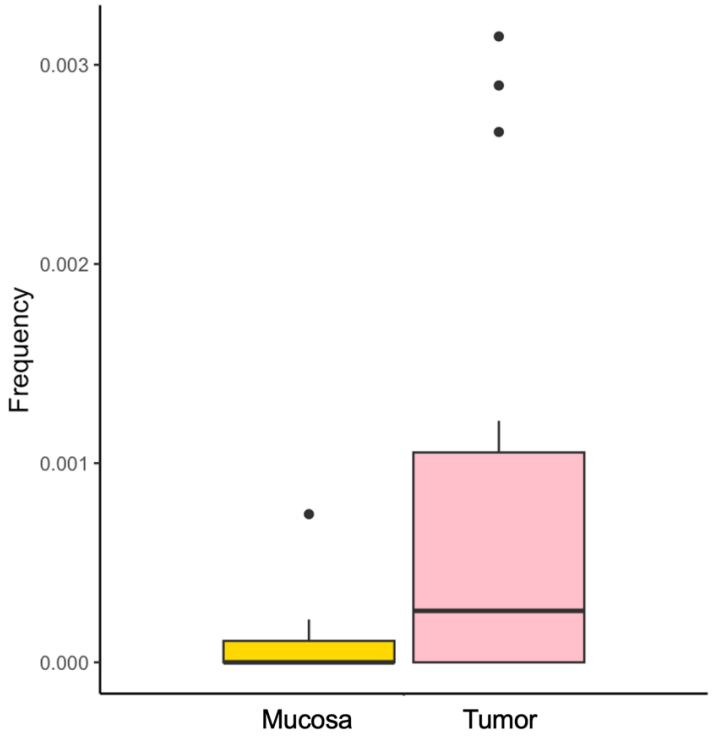

Figure 53. Relative abundance of plasmacitoid dendritic cells in the scRNA seq cohort. Tumor (n=15), unaffected mucosa (n=7).

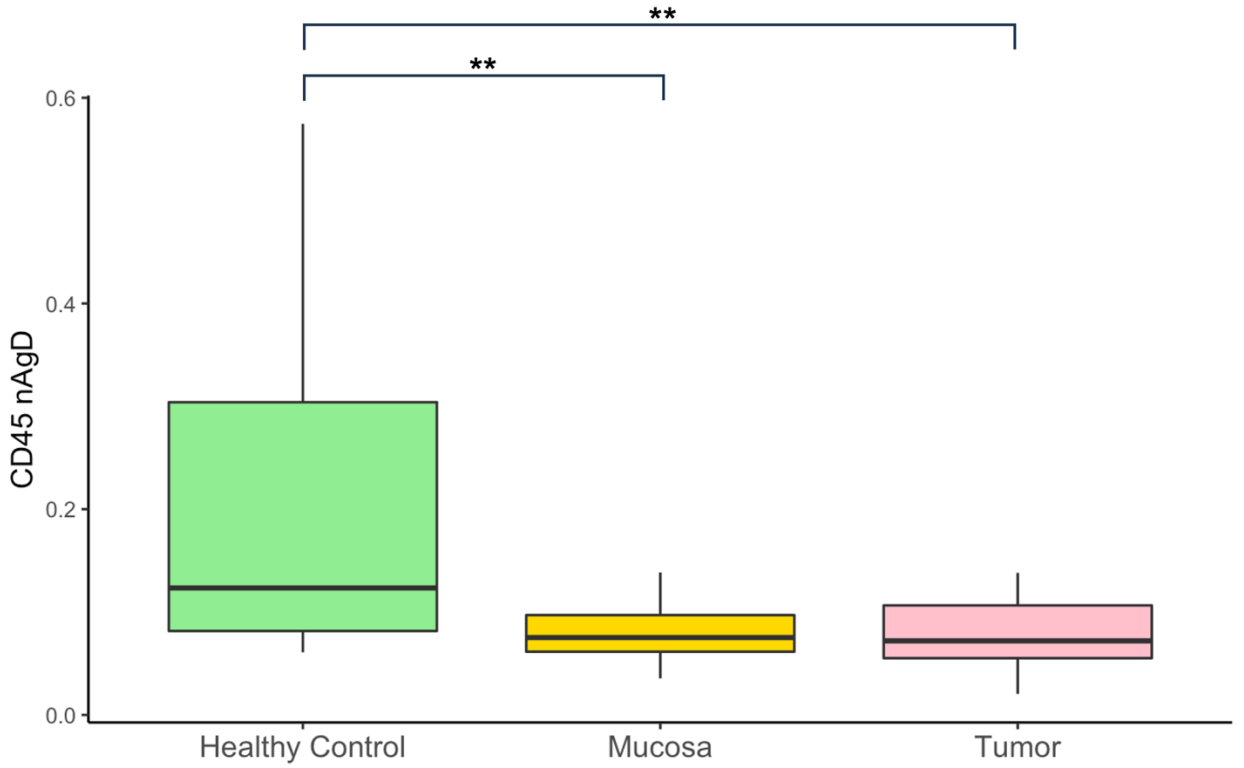

Figure 54. Normalized antigenic density (nAgD) of CD45 on the surface of plasmacitoid dendritic cells in the flow cytometry cohort. Tumor (n=28), unaffected mucosa (n=28), healthy control (n=16).

The number of conventional dendritic cells increases while their surface abundance of CD45 decreases in colorectal cancer

Conventional dendritic cells (cDCs) play an important role in colonic mucosa by regulating inflammatory responses independently from T lymphocytes. Depending on the mechanism of activation, they can trigger a strong inflammatory process or its resolution (222). Furthermore, they are the most efficient antigen presenting cells and can perform cross-presentation. Due to their interaction with T lymphocytes, they can influence T lymphocyte activation, differentiation, and proliferation (223). Thus, making them critical players in the tumor microenvironment. Figure 55 shows that the number of cDCs is significantly higher in the tumor. Accordingly, a similar trend is observed in the scRNA seq data (Figure 56).

CD45 is essential for dendritic cells to produce pro-inflammatory cytokines (224,225). Figure 57 shows that the amount of CD45 on the surface of cDCs is significantly higher in healthy controls than in the tumor or the unaffected mucosa. Overall, this result and the previous one indicate that although the number of cDCs is higher in the tumor, they are not activated to fight the tumor.

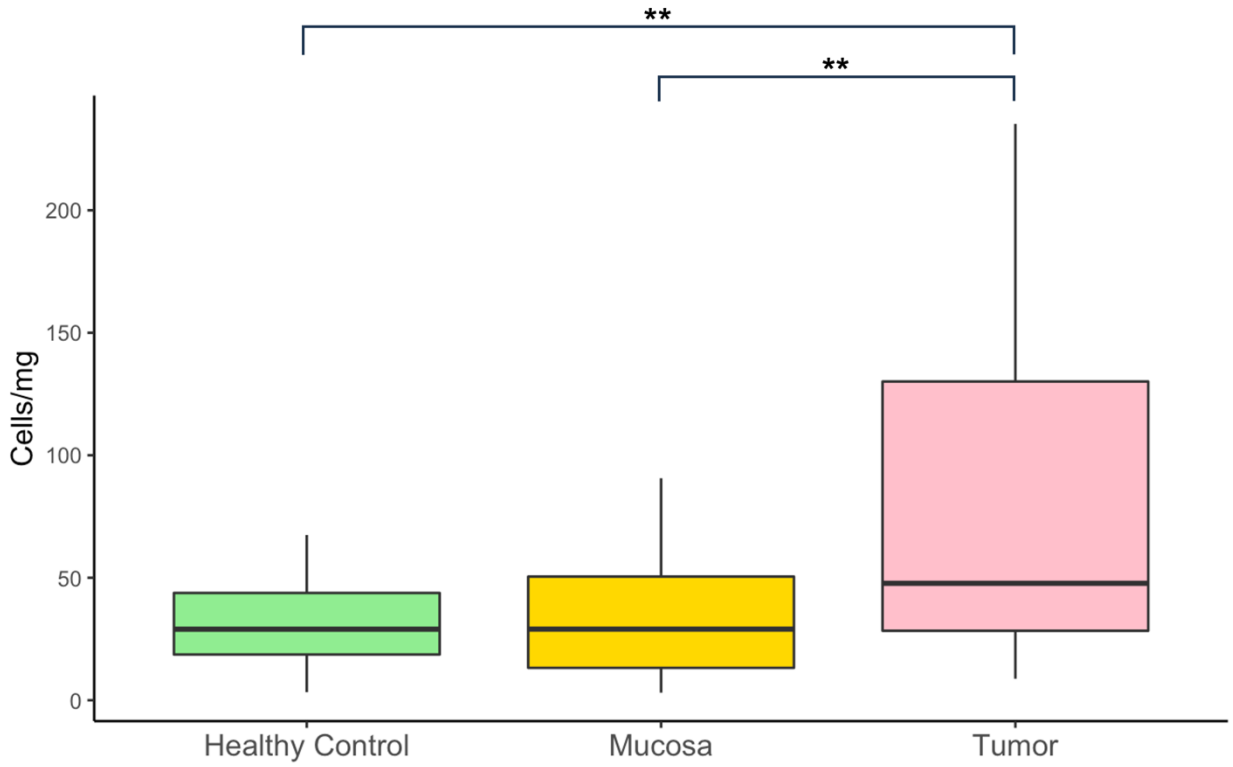

Figure 55. Number of conventional dendritic cells per mg of tissue in the flow cytometry cohort. Tumor (n=28), unaffected mucosa (n=28), healthy control (n=16).

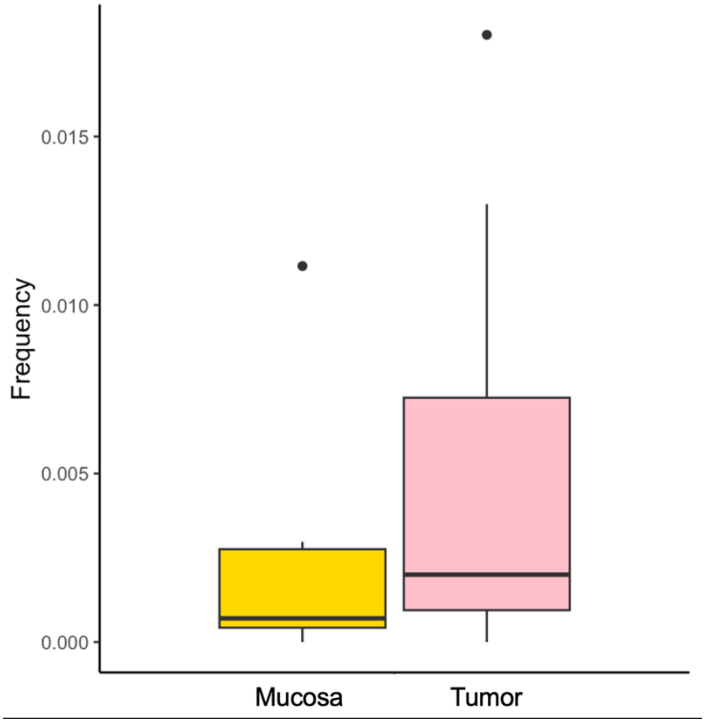

Figure 56. Relative abundance of conventional dendritic cells in the scRNA seq cohort. Tumor (n=15), unaffected mucosa (n=7).

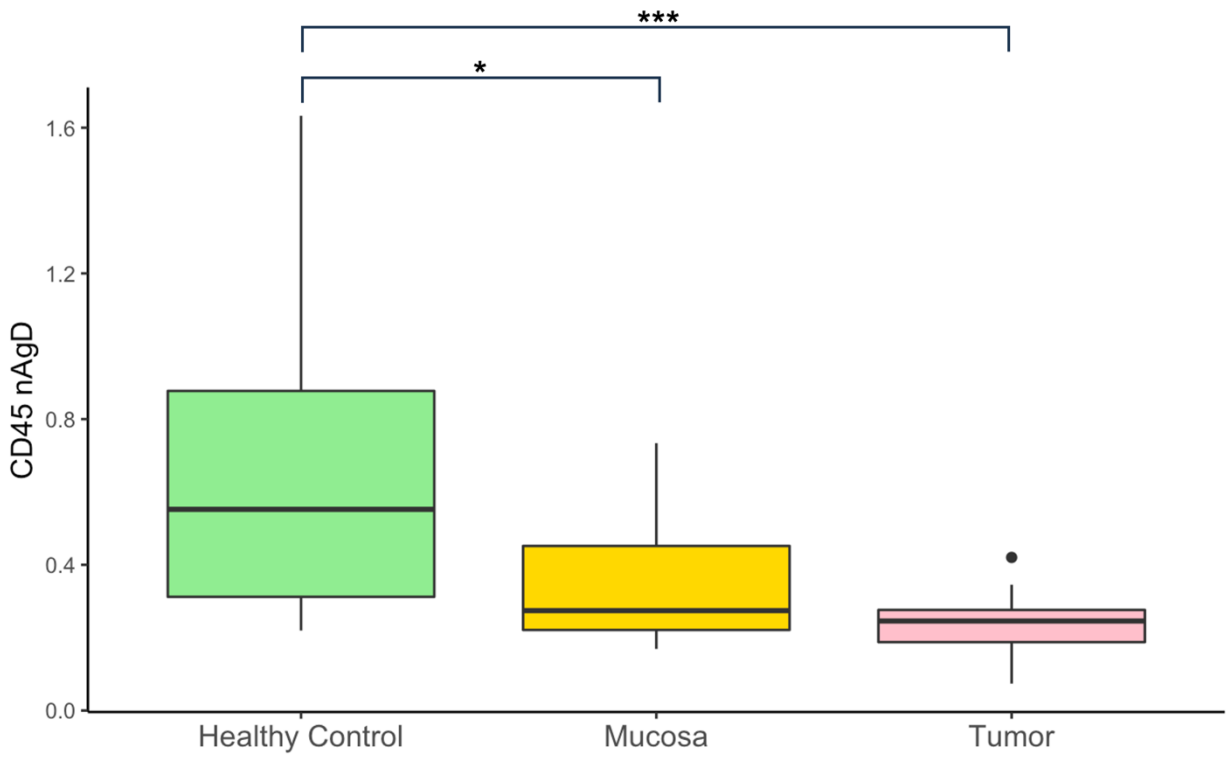

Figure 57. Normalized antigenic density (nAgD) of CD45 on the surface of conventional dendritic cells in the flow cytometry cohort. Tumor (n=28), unaffected mucosa (n=28), healthy control (n=16).

The number of mast cells increases in colorectal cancer while downregulating CD44, CD117, and CD200 but keeping stable CD45, CD203c, and FceRI

Mast cells are important in the management of the extracellular matrix. However, their role in CRC is still controversial. On the one hand, they promote extracellular matrix deposition through the release of tryptase and angiogenesis through VEGF (226). On the other hand, they trigger endoplasmic reticulum stress and the unfolded protein response selectively in CRC cells which suppresses CRC development in vivo (227). Besides, mast cells also contribute to innate immune responses (228). Interestingly, some authors found the number of mast cells as a positive prognostic marker (227,229) while others found them as negative prognostic markers (230,231). Figure 58 illustrates that the number of mast cells increases in the tumor, and this is corroborated by the scRNA seq data (Figure 59).

Unlike in the other cells analyzed, CD45 has a broader role in mast cells, and it is not restricted to the activation of a pro-inflammatory or effector phenotype. Unfortunately, there are very few studies about the role of CD45 in mast cells. CD45 oversees the fine-tuning of mast cells responses, for example after FcεRI activation (232). Additionally, it is important in the response to stem cell factor and IL-3 which are critical for their proliferation and survival (233). In fact, the

surface abundance of CD45 increases in mastocytosis, which further supports its role in mast cell proliferation and survival (234). Figure 60 shows that there are no significant differences in the abundance of CD45 on the surface of mast cells in the tumor, unaffected mucosa, or healthy controls. As mentioned before, this is probably because CD45 plays a more general role in mast cells than in the other cells studied and thus it is important for both pro and anti-tumoral responses.

FcεRI is the high affinity receptor for IgE. It is frequently employed for the identification of mast cells, and it also triggers mast cells degranulation in allergic reactions (235). Figure 61 indicates that there are no changes in the surface abundance of FcεRI between tumor, unaffected mucosa, and healthy controls. This was expected since IgE-FcεRI activation is not a relevant mechanism in CRC.

CD203c is a protein found in the membrane of the granules. During the degranulation, the fusion of the granule membrane with the plasma membrane releases the granule content to the extracellular space. Therefore, the CD203c of the granule membrane is now in the plasma membrane and it can be used to estimate mast cell degranulation (236,237). Figure 62 shows that there is no difference in the surface abundance of CD203c among tumor, unaffected mucosa, and healthy controls. This indicates that there are no differences in the degranulation rate. Since mast cells play important roles both in normal mucosa and the tumor, probably the difference is in the content of the granules.

CD117 is the receptor of stem cell factor, being critical protein for mast cell differentiation. Besides, in fully differentiated mast cells it promotes their survival, collaborates with other receptors to enhance their signaling, and can increase the upregulation of certain cytokines (238,239). Figure 63 indicates that the abundance of CD117 on the surface of mast cells decreases in the tumor, which may suggest a reduced survival. However, Figure 58 demonstrates that the number of mast cells increases in the tumor. This apparent contradiction has 3 possible explanations. Firstly, CRC tumors are enriched in IL-10 (240), which downregulates CD117 expression (238). In this case, other pathways like the IL-3 signaling pathway could provide the pro-survival signals (239). Interestingly, IL-10 enhances IL-3 signaling in mast cells (239). Secondly, since CD117 is quickly internalized after the binding of the stem cell factor (238), the excess of stem cell factor in the TME may be reducing the CD117 available on the membrane of mast cells while increasing its activity. Noteworthy, the serum levels of IL-3 and stem cell factor are increased in CRC patients (241), and then these two mechanisms could be running in parallel. Finally, the last possibility is a high turnover rate of mast cells in the tumor. However, since mast cell progenitors are very infrequent in peripheral blood (242) and they were not found in this study, this possibility is remote.

CD123 is the α chain of the IL-3 receptor, being this a molecule usually overexpressed in hematologic tumors (243). In mast cells, IL-3 provides strong pro-survival signals (244). Besides, it also contributes to mast cell differentiation although it is unclear if it requires the synergistic action of the stem cell factor (244). Figure 64 shows a trend in which CD123 seems to increase in

the tumor. Unfortunately, the results are not statistically significant. However, it should be taken into account that CD123 was analyzed in considerably less patients than all the other markers.

CD44 is the receptor for hyaluronic acid, although it can also bind collagen and metalloproteases (245). Its biology is very complex, and it changes in different cell types. In mast cells, in normal conditions it binds high molecular weight hyaluronic acid keeping the mast cell attached to the extracellular matrix (246). When there is matrix deposition, the high molecular weight hyaluronic acid is broken down to low molecular weight units. Then, the CD44 of mast cells binds this low molecular weight hyaluronic acid molecules and they are endocytosed (246). This allows the quick and efficient removal of low molecular weight hyaluronic acid. If the low molecular weight hyaluronic acid is not removed, it activates other immune cells triggering a strong Th1 response (246). Figure 65 displays the abundance of CD44 on the surface of mast cells, which is higher in the healthy control, then in the unaffected mucosa, and finally the lowest levels occur in the tumor. Since matrix deposition is a very common event in the TME (247), it seems that most of CD44 is endocytosed with low molecular weight hyaluronic acid.

CD200 is a controversial immunomodulatory molecule that has attracted great interest in recent times. It was initially described as an immune checkpoint that inhibits myeloid cells and T lymphocytes (248). Besides, since many tumors are $CD200^{+}$, the blocking of the CD200-CD200R axis is currently under testing in clinical trials (248). However, other authors have seen that the blockade of the CD200-CD200R axis dramatically enhances the expansion of macrophages and MDSCs, thus leading to tumor progression (249). Additionally, the CD200-CD200R axis seems to be important for dendritic cells to phagocytose dead cancer cells and promote an antitumoral response (249). It looks like the outcome of the CD200-CD200R axis strongly depends on TME interactions and more research is needed to completely understand its function. Interestingly, the abundance of CD200 on the surface of mast cells decreases in the tumor (Figure 66). Although the role of CD200 on mast cells is currently unknown, it may be possible that the reduction of surface CD200 on the surface of mast cells could contribute to the proliferation of macrophages and MDSCs. Maybe, the mast cell is more polarized towards its non-immunological functions due to the huge extracellular matrix remodeling. Finally, other authors have seen that the ectodomain of CD200 can be shedded by metalloproteases and activate CD200R in distant cells in a paracrine manner (250). Anyway, the fact that such significant p-values were obtained with a very low sample size indicates that this may be an interesting topic to pursue.

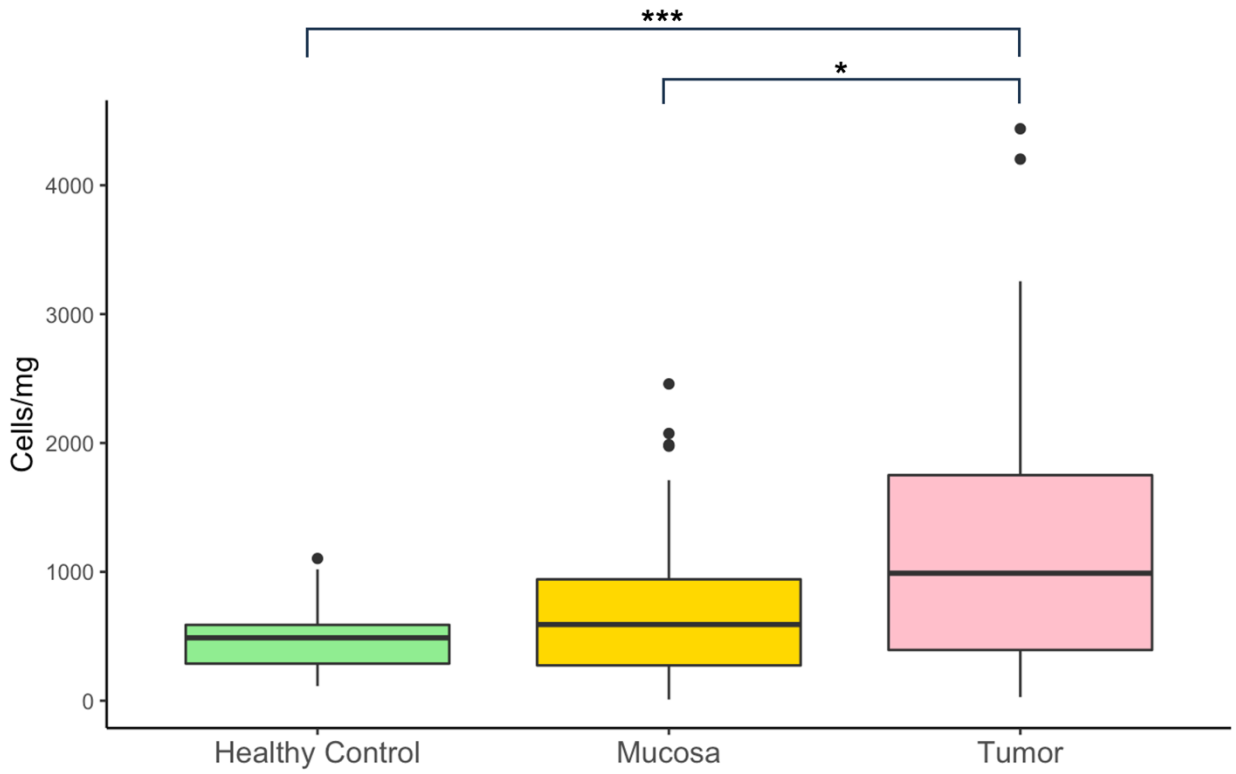

Figure 58. Number of mast cells per mg of tissue in the flow cytometry cohort. Tumor (n=54), unaffected mucosa (n=54), healthy control (n=20).

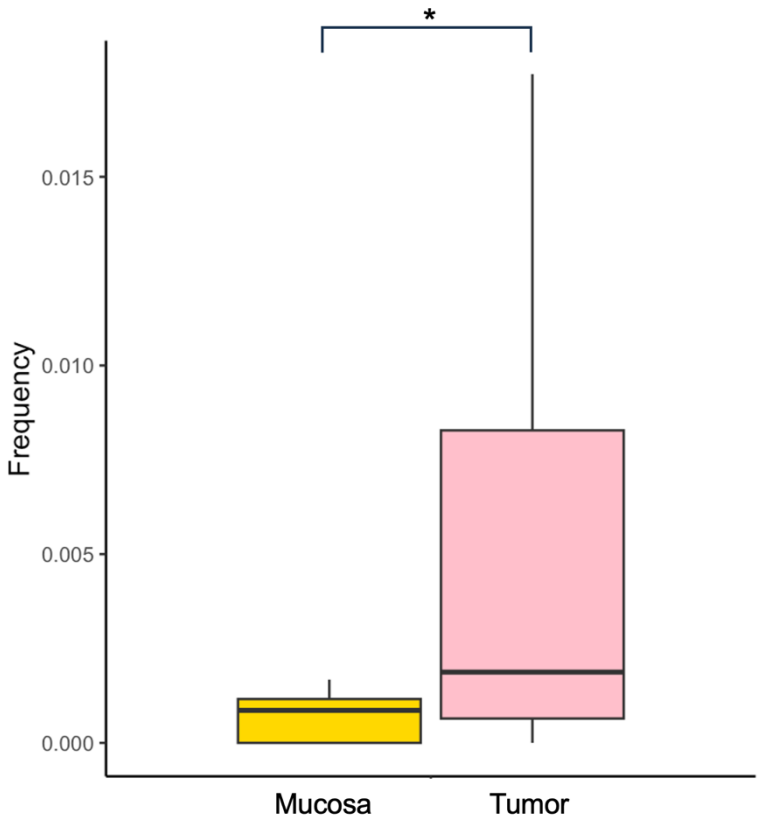

Figure 59. Relative abundance of mast cells in the scRNA seq cohort. Tumor (n=15), unaffected mucosa (n=7).

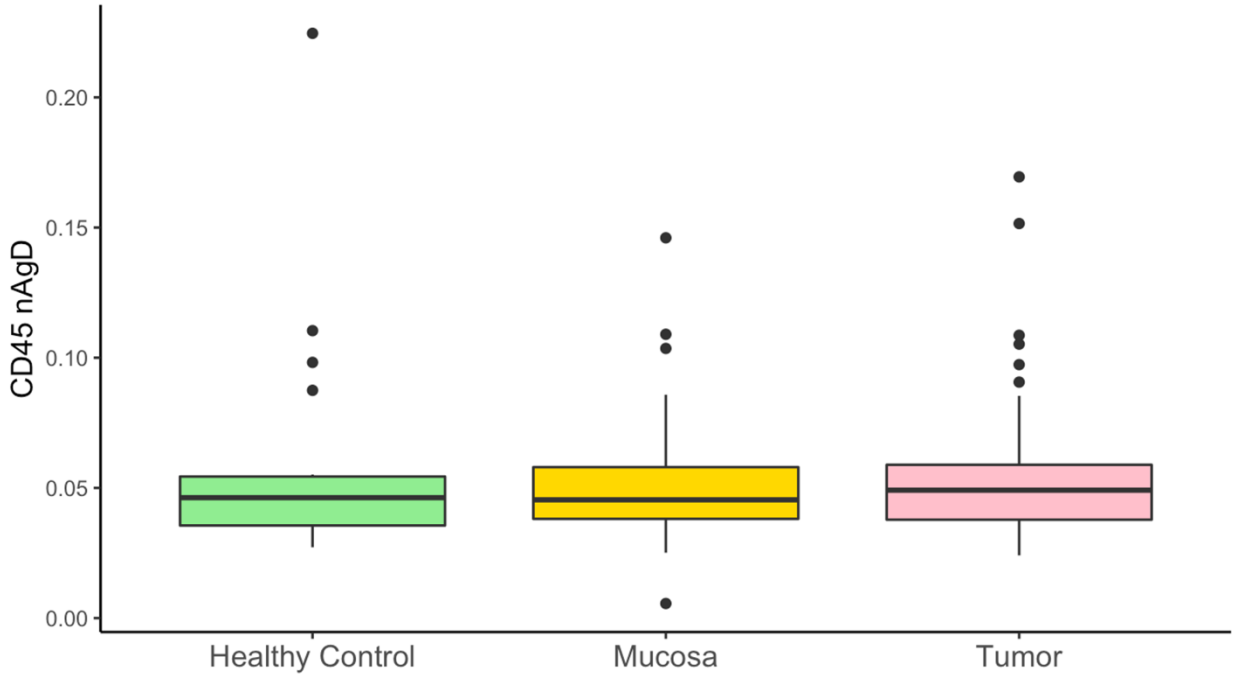

Figure 60. Normalized antigenic density (nAgD) of CD45 on the surface of mast cells in the flow cytometry cohort. Tumor (n=54), unaffected mucosa (n=54), healthy control (n=20).

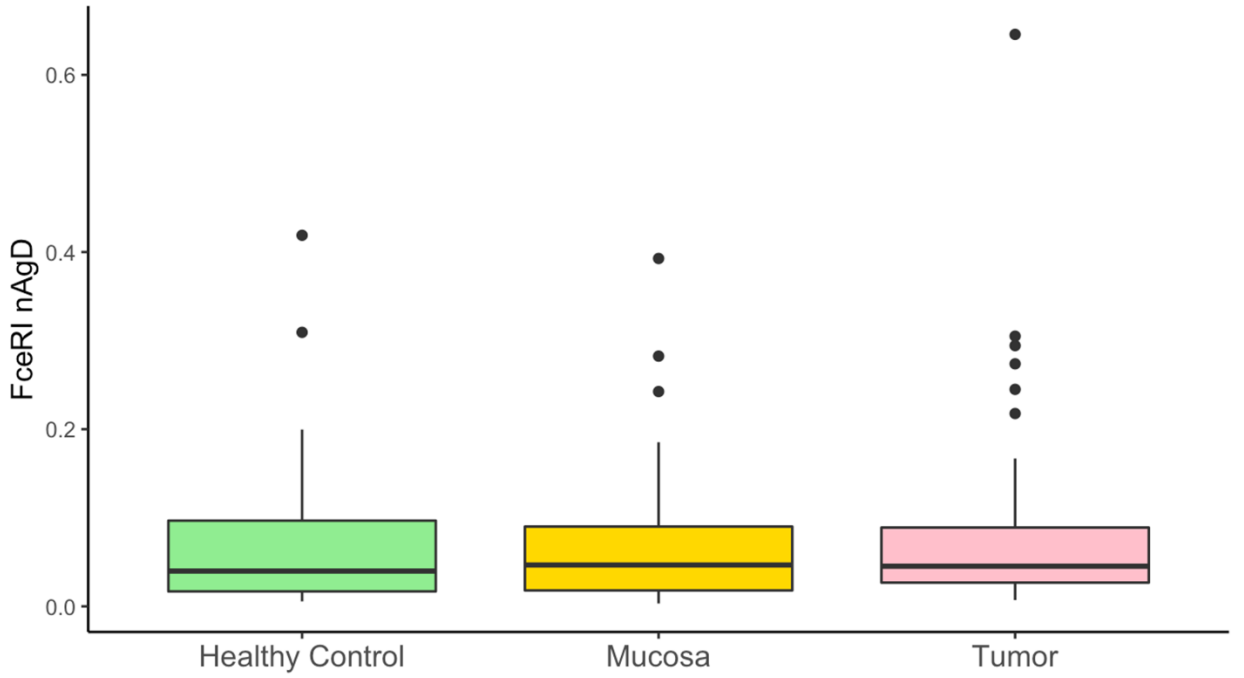

Figure 61. Normalized antigenic density (nAgD) of FceRI on the surface of mast cells in the flow cytometry cohort. Tumor (n=44), unaffected mucosa (n=44), healthy control (n=19).

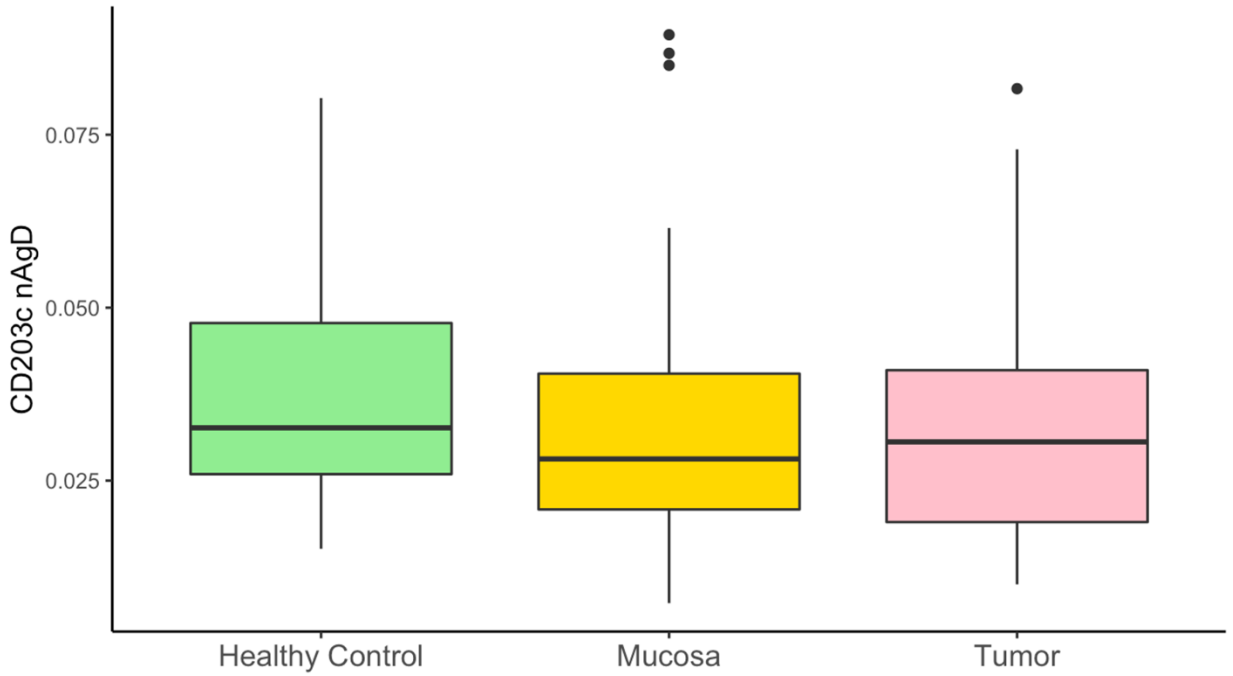

Figure 62. Normalized antigenic density (nAgD) of CD203c on the surface of mast cells in the flow cytometry cohort. Tumor (n=44), unaffected mucosa (n=44), healthy control (n=18).

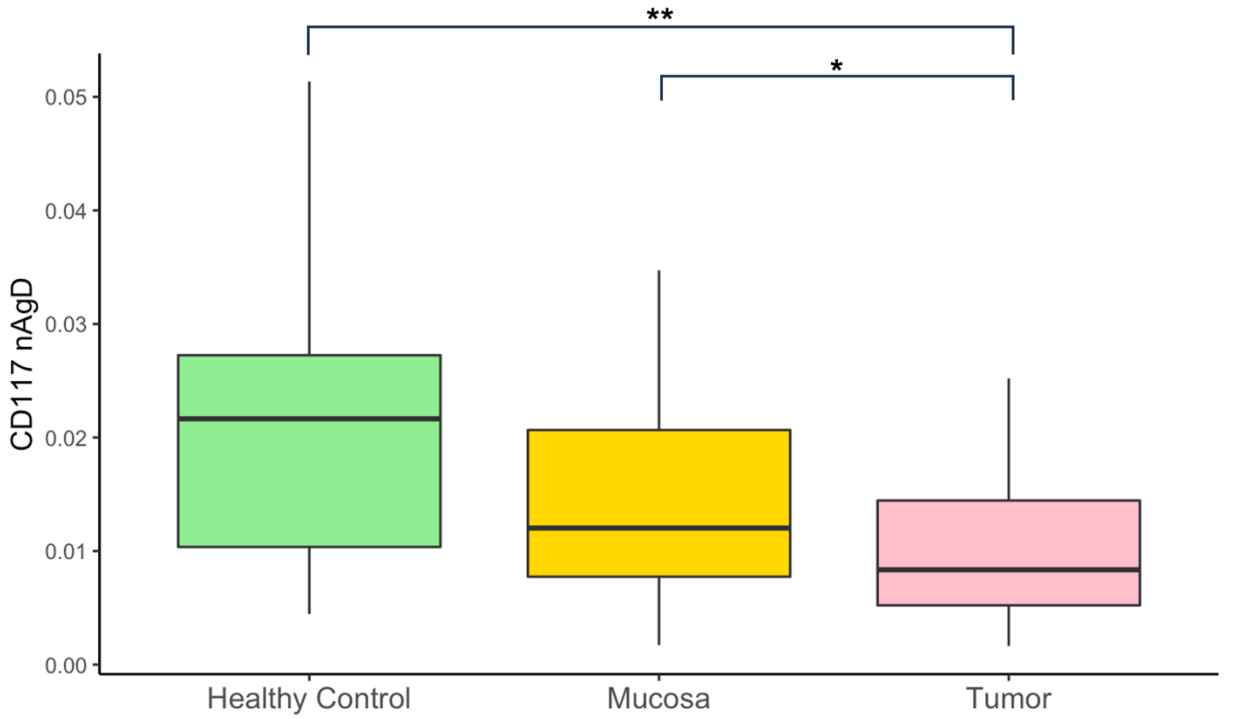

Figure 63. Normalized antigenic density (nAgD) of CD117 on the surface of mast cells in the flow cytometry cohort. Tumor (n=54), unaffected mucosa (n=54), healthy control (n=20).

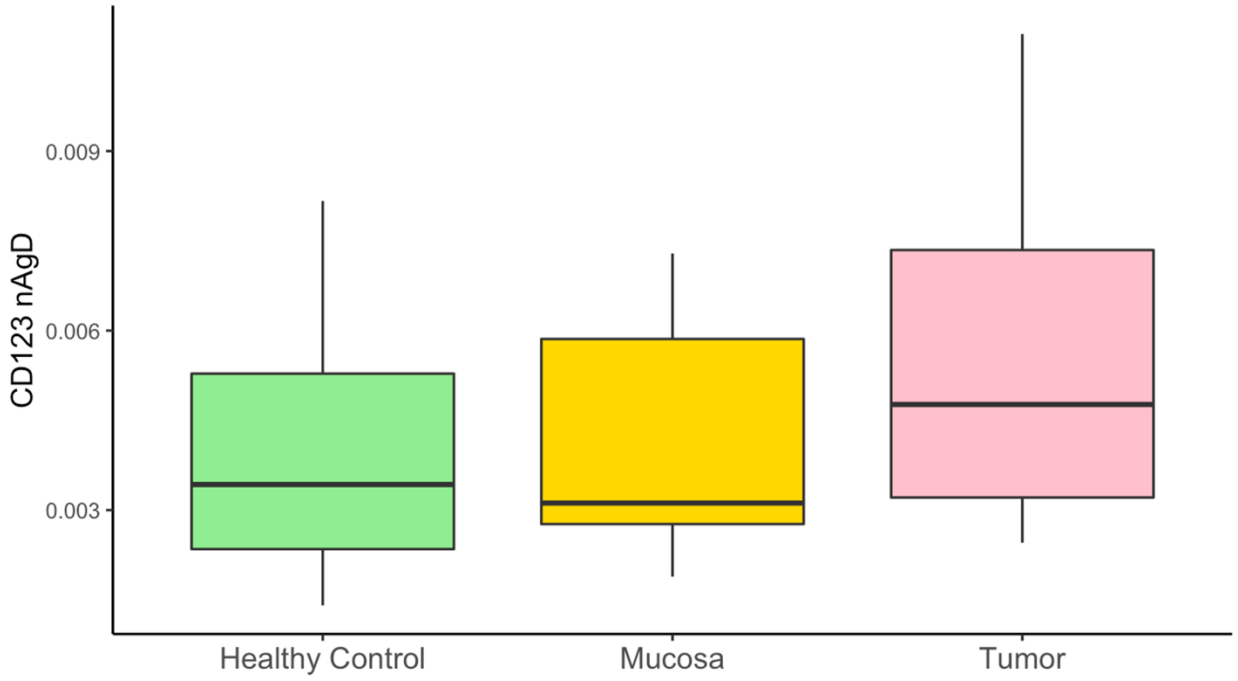

Figure 64. Normalized antigenic density (nAgD) of CD123 on the surface of mast cells in the flow cytometry cohort. Tumor (n=12), unaffected mucosa (n=12), healthy control (n=10).

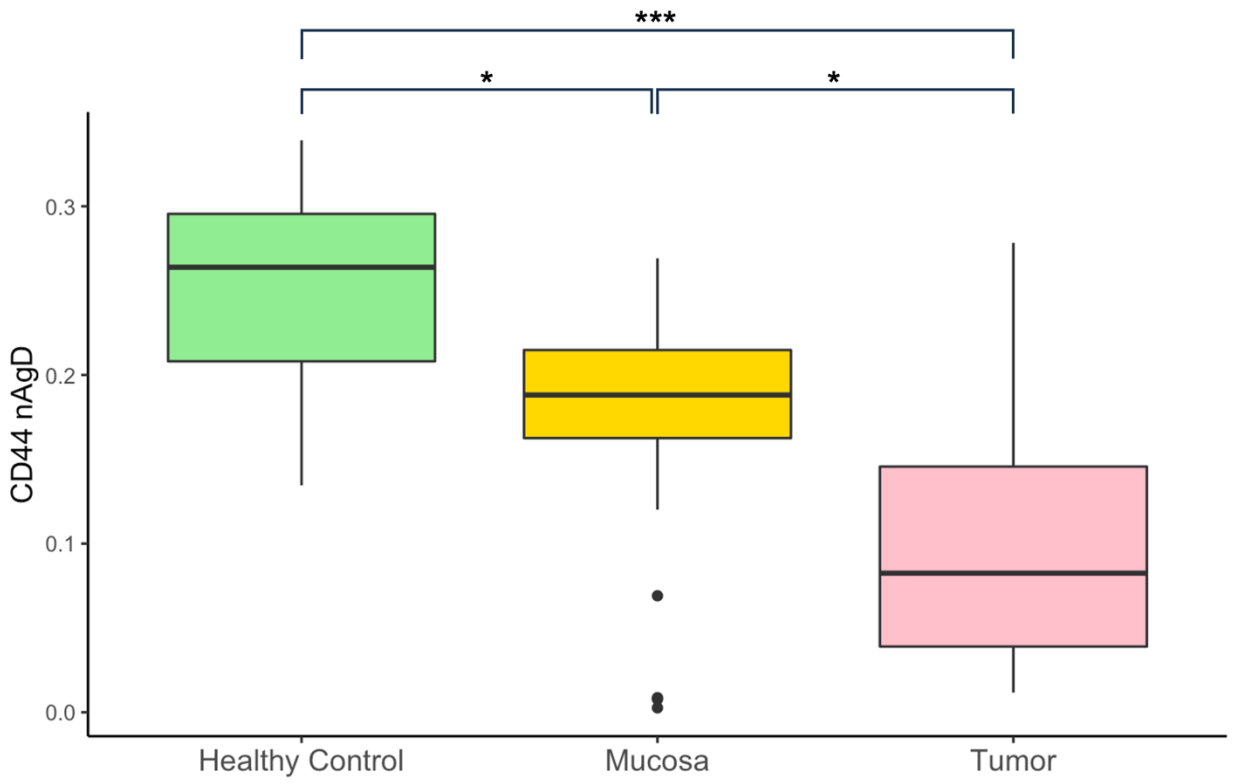

Figure 65. Normalized antigenic density (nAgD) of CD44 on the surface of mast cells in the flow cytometry cohort. Tumor (n=22), unaffected mucosa (n=22), healthy control (n=10).

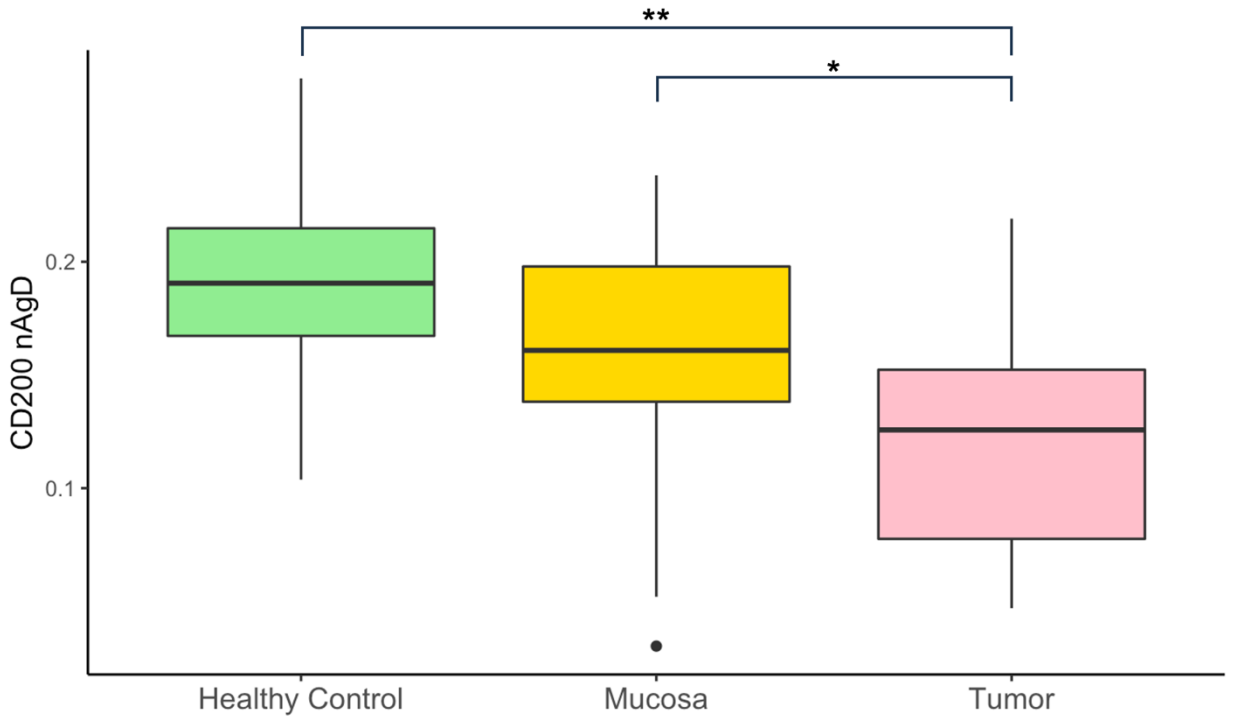

Figure 66. Normalized antigenic density (nAgD) of CD200 on the surface of mast cells in the flow cytometry cohort. Tumor (n=22), unaffected mucosa (n=22), healthy control (n=10).

Discussion

Traditionally, the composition of the TME has been studied through histological techniques. However, this technique provides a limited amount of cellular information since each slide only includes hundreds of cells and a few markers (251). Besides, the quantification is difficult sometimes and involves some degree of subjectivity from the pathologist which may lead to reproducibility problems. On the other hand, flow cytometry is a powerful tool that allows for the analysis of thousands of individual cells in a short amount of time (251). Besides, you can use more markers than in histological techniques, which allows the identification of more cell types in the same experiment or a better accuracy in cell type identification (251). This technique provides a more comprehensive understanding of the cellular composition of the tumor microenvironment and allows for quantitative analysis with greater accuracy and reproducibility. In the past, some results that seemed to be very straightforward using histological techniques were found completely wrong when analyzed through flow cytometry (252). Therefore, flow cytometry has emerged as a valuable approach to validate the findings obtained through traditional histological techniques. Nevertheless, performing high-quality flow cytometry requires preparing a good cell suspension (251). This is not a problem with blood samples but could be very challenging with solid tissues. In the beginning of this project, the sample digestion was performed just with collagenase, but the suspensions obtained had poor-quality. The problem was the high amount of glycosaminoglycans in the extracellular matrix, and it was solved by adding hyaluronidase, chondroitinase and heparinase to the digestion mix. This was critical since there was considerable variability in the amount of glycosaminoglycans between patients as well as between unaffected mucosa and tumor within the same patient. After this addition to the initial protocol, the suspensions obtained had high-quality and allowed us to perform precise quantifications.

In the scRNA seq data we observed that the number of pDCs and cDCs follows the same trend than in the flow cytometry data, but the differences are not statistically significant. This problem is probably is due to the reduced sample size of the scRNA seq cohort compared to the flow cytometry cohort. Thus, if the sample size is increased in the scRNA seq cohort, the results will probably become significant. Similarly, the abundance of CD123 on the surface of mast cells shows a clear trend but it is not statistically significant. Here, the sample size was by far the smallest of all the markers tested. In both cases, the data did not fit the criteria for the use of parametric tests. Therefore, the analysis was performed using non-parametric tests, which are more robust but with them it is more difficult to reject the null hypothesis.

The present study indicates immunological alterations in the mucosa near colorectal cancer (CRC) compared to healthy controls. However, these alterations are less significant than those observed in the tumor. The unaffected mucosa of CRC patients showed significantly higher levels of neutrophils and lower levels of eosinophils compared to healthy controls. Interestingly, while eosinophils dominate in healthy mucosa, they are replaced by neutrophils in the TME. This

observation suggests a potential role for neutrophils in tumor growth and a potential role for eosinophils in tumor surveillance.

The role of neutrophils in colorectal cancer

Colon inflammation is required for tumor initiation in CRC. However, the specific mechanisms that determine if this colonic inflammation will progress to CRC or not are not completely understood. To shed light on this topic, researchers are studying how patients with inflammatory bowel disease (IBD) are at a higher risk for developing CRC compared to the general population (253). IBD includes a heterogeneous group of diseases characterized by chronic inflammation of the mucosa of the digestive system that affects mainly the colon (253). This inflammation recruits a large number of neutrophils that are pivotal for the maintenance of the inflammatory environment and the progression to CRC (254). Inhibition of FOXO3 in neutrophils induces metabolic changes and low-grade inflammation, resulting in increased tumor growth and invasion (255). Besides, neutrophils release neutrophil-activated serine proteases (NSPs) that cleave insulin-like growth factor-binding protein 3 (IGFBP-3), thereby inhibiting its signal transduction. This cleavage of IGFBP-3 contributes to the progression of IBD to colon cancer. Noteworthy, pharmacological inhibition of NSPs reduces inflammation and tumorigenesis (256). In short, neutrophils are essential for the transition from an inflamed microenvironment to a tumoral microenvironment. Although only a small fraction of CRCs come from IBD patients, since inflammation is a hallmark of cancer (257) it is reasonable to think that neutrophils will be important players in this inflammation-tumor transition in sporadic CRC too.

When CRC is established, tumor cells with stem cell-like properties release tumor RNA-laden exosomes that arrive in the bone marrow, where they promote neutrophil survival and prime them to become tumor-promoting cells. These primed neutrophils are then recruited to the tumor microenvironment thereby promoting tumor growth (258).

The interaction between neutrophils and CRC evolves as the tumor progress. In the early stages, neutrophils release miR-155, which downregulates RAD51 in tumor cells and impairs homologous recombination. Thus, preventing loss of heterozygosity and attenuating tumor growth (259). However, in advanced stages, neutrophils release miR-23a, which downregulates Lamin B1 promoting replicative stress and accumulation of double-strand breaks. This leads to non-homologous end joining (NHEJ), which allows tumor survival and progression (259).

Neutrophil plasticity in the tumor environment is regulated by interferon-β and transforming growth factor (TGF)-β signaling. The former promotes anti-tumoral inflammation and cytotoxicity while the latter causes immunosuppression and tumor growth (260).

Tumor-associated neutrophils (TANs) cytotoxicity against tumor cells is mainly due to the generation of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide. ROS-induced cell killing depends on tumor cell expression of TRPM2, an H2O2-dependent Ca2

channel, resulting in a lethal calcium influx (261). In addition, TAN can inhibit metastatic seeding in the lungs by producing hydrogen peroxide. Neutrophils also release reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, which can cause cancer cell death (261). Nevertheless, since CRC displays an increased expression of TGF-β compared to normal mucosa, the immunosuppressive phenotype predominates (260).

Immunosuppressive TANs release enzymes such as myeloperoxidase (MPO), neutrophil elastase (NE), and matrix metalloproteinase (MMP) to remodel the extracellular matrix, stimulate angiogenesis, and promote cancer migration and invasion (261). Importantly, TGF-β inhibition increased TAN cytotoxicity, reduced metastatic chemoattractant secretion, promoted apoptosis in CRC cells, and inhibited tumor cell migration (260). This effect was partly due to the inhibition of PI3K/AKT signaling in TAN and partly to the inhibition of TGF-β/Smad signaling in tumor cells (260). Noteworthy, neutrophil depletion reduces the effect of anti-TGF-β therapy (260). Activation of NOTCH1 signaling in murine intestinal epithelium induces highly invasive metastasis in a KrasG12D-driven serrated carcinoma model. The tumor microenvironment generated by epithelial NOTCH1 signaling promotes metastasis through TGFβ-dependent neutrophil recruitment. Inhibition of neutrophil recruitment by therapeutic agents prevents metastasis (262).

Moreover, TANs can also act as immunomodulators. Interestingly, TANs are the most potent inhibitors of T-cell activity in CRC. This is achieved through neutrophil-secreted metalloproteinases which activate latent TGF-β and suppress T cells. In vitro studies with neutrophils from patients with CRC confirmed their ability to suppress T cells using TGF-β, and a gene expression data set showed that CRCs with combined neutrophil infiltration and TGF-β activation had the lowest T cell activity (263). Therefore, immunosuppressive TANs and TGF-β establish a feed-forward loop in which the vast majority of TANs become immunosuppressive and the amount of TGF-β available in the TME increases.

Neutrophil extracellular traps (NETs) are networks composed of nuclear DNA-derived structures and neutrophil-derived proteins that are thrown outside of the neutrophil to trap other cells. In the beginning, it was thought that NETs were only used to trap microbes, but later it was seen that they can trap bigger cells too (264). Citrullinated NETs in CRC were significantly associated with high histopathological tumor grade and lymph node metastasis (265). Purified NETs induce an epithelial-mesenchymal transition (EMT)-like process in CRC cells (265). Moreover, NETs trap tumor cells in the liver and lung, increasing adhesion and metastasis (266,267). The NET-associated carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) plays a critical role in this interaction. Blocking or knocking down CEACAM1 reduces adhesion, migration, and metastasis of colon cancer cells (266).

Overall, these studies demonstrate the significant role that neutrophils play in colorectal cancer and underscore the complex and intimate relationship between neutrophils and TME. Although it is clear that neutrophils switch from an anti-tumoral to a pro-tumoral phenotype in CRC the mechanism is not completely understood. In the present study it is described that the increased number of neutrophils in CRC compared to healthy mucosa is coupled with a decrease in the

surface abundance of CD45. As it will be discussed later, CD45 controls immune phenotype through a complex network of interactions with other membrane receptors and signal transducers. Therefore, the reduction of CD45 on the surface of neutrophils necessarily imply an important change in their regulatory network that may contribute to this switch from anti-tumoral to pro-tumoral phenotype. To the best of our knowledge, this is the first study to examine the differences in surface expression of CD45 on neutrophils between CRC and mucosa.

The role of eosinophils in colorectal cancer

The gastrointestinal tract is the main homing location for eosinophils. Therefore, they are quite abundant in colonic mucosa under normal circumstances (216). In the early stages of CRC, the pro-inflammatory milieu of the TME includes eotaxins (CCL11, CCL24, and CCL26) and ELR^+ CXC chemokines (268–270), leading to increased recruitment and survival of eosinophils compared to normal mucosa (271,272). This is increased after dying tumor cells release the high mobility group box 1 protein (HMGB1) and IL-33 (273,274).

In the TME, hypoxic conditions promote the upregulation of ICAM-1 in tumor cells (275). Besides, stress epithelial cells release IL-18, which promotes the upregulation of ICAM-1 in tumor cells (276,277). The presence of IL-18, IL-33, and HMGB1 activates eosinophils. Then, they bind ICAM-1 on tumor cells through LFA-1 and ITGAM, and secrete TNF-α, granzyme A, ROS, nitric oxide, and eosinophil extracellular traps (EETs) to kill tumor cells (277–280). Interestingly, IL-18 KO murine models display higher CRC frequency and aggressiveness and IL-33 KO models show impaired eosinophil cytotoxicity against CRC (281–283).

The effector function of eosinophils is carried out by EETs and granule release. Contrary to NETs, EETs are made of mitochondrial DNA instead of nuclear DNA and have a predominantly anti-tumoral role. Moreover, EETs are decorated with major basic protein (MBP) and eosinophil cationic protein (ECP), which have strong cytotoxic activity (284,285). Importantly, since EETs are made of mitochondrial DNA and not nuclear DNA, their release does not necessarily lead to eosinophil death, and they can be used several times (286). In addition to EETs, eosinophils release granules with cytotoxic compounds including MBP, ECP, eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN), and granzyme A. MBP and ECP have a high pI and generate cell membrane disruption, chromatin condensation, ROS synthesis, activation of caspase-3, and cell death. EPO causes oxidative stress and lipid oxidation in target cells. Finally, EDN and granzyme A degrade RNA and proteins involved in DNA repair (287).

In addition to their effector function, eosinophils can also modulate other immune cells in the tumor microenvironment. During stress or inflammation, different cells secrete GM-CSF, which activates IRF5 in eosinophils. Such induces the release of pro-inflammatory cytokines (IL-1α, IL-1β, and TNF-α) and chemokines (CCL4, CCL5, CXCL9, and CXCL10) that recruit and activate $CD8^+$ T lymphocytes (217,218). Besides, eosinophils recruit neutrophils through CXCL1 and CXCL8, and polarize macrophages to M1 phenotypes (288–290).

Eosinophils also play a role in angiogenesis in the TME. In CRC, different cell types produce an excessive amount of pro-angiogenic factors. This unbalance produces abnormal vessels that cause reduced perfusion, acidosis, and hypoxia, and inhibit the recruitment of effector leukocytes (291). Noteworthy, in murine models the administration of eosinophils normalized the vessels in the TME and increase the number of M1 macrophages and $CD8^+$ T lymphocytes promoting tumor rejection (218).

As CRC progresses, it reduces the secretion of eotaxins, which impairs the recruitment of eosinophils (292). Moreover, this is coupled with an increase of IL-10 in the TME that inhibits IRF5 signaling in eosinophils, blocking the production of pro-inflammatory cytokines and chemokines and their contribution to anti-tumoral immunity (217,218,293). Besides, the upregulation of Fas-L in CRC activates Fas in the membrane of eosinophils triggering their apoptosis. This contributes to more abnormal angiogenesis and further hypoxia, acidosis, and immunosuppression (294,295).

In summary, eosinophils act as effector cells in tumor tissues by releasing cytotoxic molecules and exerting their anti-tumor effects through mechanisms such as oxidative burst, cytokine secretion, and modulation of immune cell recruitment and activation. This multifaceted role of eosinophils in the tumor microenvironment highlights their potential as therapeutic targets for enhancing anti-tumor immunity and normalizing tumor vasculature to improve the efficacy of cancer treatments. However, further research is needed to fully elucidate all the anti-tumoral mechanisms in which eosinophils take part.

The present study shows a decrease on both eosinophil number and CD45 abundance on eosinophil surface. From both results, the decrease in number seems much more relevant. However, its interesting that despite following different population dynamics than neutrophils, pDCs, and cDCs, eosinophils share with all of them the decrease in CD45.

Both eosinophils and neutrophils are generally recruited to inflammatory microenvironments. In CRC, as the tumor progresses it modifies the inflammation of the microenvironment from immunostimulatory or anti-tumoral to immunosuppressive or pro-tumoral. In this switch, the innate immune cells are polarized to this immunosuppressive phenotype, except eosinophils, which are excluded from the TME. Eosinophils can acquire immunosuppressive features in other contexts (296,297), so there is no clear reason for this exclusion. Remarkably, this study shows that this exclusion occurs also in the nearby unaffected mucosa. Accordingly, the number of eosinophils in the peritumoral mucosa is a favorable prognostic factor (298).

The role of dendritic cells in colorectal cancer

Dendritic cells have been extensively studied in solid tumors. However, despite their different roles, the distinction between pDCs and cDCs is not routinely performed. Interestingly, unlike tolerant cDCs, immunosuppressive pDCs do not necessarily have an immature phenotype (299).

In the present study, the number of cDCs doubles the number of pDCs. Therefore, the papers that report the presence of dendritic cells without specifying the subtype may be biased toward cDCs.

The function of pDCs has been extensively studied in viral infections and autoimmune diseases, but their role in TME has been overlooked. In CRC, pDCs have been poorly studied and their role remains controversial. Several reports have shown that pDCs are a favorable prognostic marker (300) and that their loss accelerates the transition from colitis to CRC through the recruitment of MDSCs (301). In contrast, other authors found that pDCs were severely compromised in their anti-tumoral and pro-inflammatory capability due to TLR7 tolerance (302). Besides, the depletion of pDCs significantly improved the response to vaccines against CRC antigens (303). In the present study, it was observed that in pDCs there is a decrease in the amount of surface CD45 in unaffected mucosa and tumor compared to healthy controls, suggesting an impaired ability to mount effective antitumor and proinflammatory responses. The beneficial effect in preventing the progression from colitis to colon cancer may be explained by a mechanism similar to that of neutrophils. The role of pDCs in CRC may be anti-tumoral or pro-tumoral, depending on the TME. At early stages, an antitumor phenotype predominates, explaining how pDCs prevent the transition from colitis to colon cancer. However, as the tumor progresses, it creates a feed-forward loop that polarizes pDCs to an immunosuppressive phenotype. Therefore, the role of pDCs in colorectal cancer remains complex and context-dependent, with potential implications for both tumor progression and therapeutic interventions.

The analysis of other solid tumors in humans and mice has shown that tumor-infiltrating pDCs primarily support the immunosuppressive environment of the TME (304). TGF-β has been identified as a key cytokine that inhibits IFN-α production in tumor-associated pDCs (305). Likewise, IL-3 stimulation contributes to enhancing the immunosuppressive phenotype of pDCs (306). This phenotype includes enhancing angiogenesis and suppressing T lymphocyte proliferation through upregulation of immune checkpoints (299). In addition, pDCs in the TME induce apoptosis of innate lymphoid cells group 3 (ILC3), which play an important anti-tumoral role in the TME (307).

In melanoma, pDCs express OX-40L and ICOSL promoting futile inflammation and differentiation of T lymphocytes into Th2 and Tregs (308). Besides, in lymph nodes and skin metastasis, there is a significant infiltration of a $LAG3^{+}$ subset of pDCs. This LAG3 induces a pDC activation that downregulates IFN-α production but promotes IL-6 secretion, suggesting that this alternative activation pathway plays an important role in establishing an immunosuppressive microenvironment (309).

In breast cancer, pDCs promote Th2 and Treg differentiation (310). Noteworthy, pDC depletion in glioma and breast cancer has been shown to reduce tumor burden, metastasis, and infiltration of Tregs in mouse models (311,312). Moreover, pDCs in ovarian cancer TME release IL-8, which is a potent chemoattractant and activator of neutrophils (313). Overall, despite the controversy surrounding the role of pDCS in CRC, there is accumulating evidence suggesting that pDCs in solid tumors exhibit an immunosuppressive phenotype and contribute to tumor progression.

From all professional antigen-presenting cells, cDCs are the most efficient and potent when interacting with T lymphocytes. Under normal conditions, when there is no threat, they are not activated but they present innocuous antigens to T cells. Such includes auto-antigens and harmless environmental antigens (like those derived from the microbiota) (314,315). Importantly, in this presentation process, cDCs do not upregulate co-stimulatory molecules (like CD80 and CD86). Thus, if a T cell recognizes the antigens presented by these cDCs, it will receive a "do not activate" signal and become unresponsive or even tolerogenic. This process is critical to establish peripheral tolerance (314,315).

On the other hand, if cDCs encounter stress or danger signals (PAMPs or DAMPs), they activate TLR signaling pathways and undergo maturation. This results in the upregulation of co-stimulatory molecules and the secretion of pro-inflammatory cytokines. Thus, if the T lymphocyte recognizes an antigen in this context, it would be activated and can initiate an immune response against that antigen (314,315). As it will be discussed below, CD45 is an important modulator of TLR-mediated signaling transduction, which suggests that the CD45 decrease in the surface of cDCs may be a relevant mechanism to evade cDC function.

Importantly, while other immune cells rely on T-lymphocyte-derived cytokines to induce immune responses or tolerance, cDCs have the unique ability to directly sense the presence or absence of threats and initiate either immune responses (both innate and adaptive) or induce tolerance (316). Noteworthy, cDCs can be further subdivided into cDC1 and cDC2. These two subpopulations follow different and independent differentiation trajectories and have different functional phenotypes (316). The main characteristic of cDC1 cells is their high capacity for antigen cross-presentation (317). This means that they can phagocytose extracellular antigens and present them to $CD8^+$T lymphocytes through MHC class I (318). This process is crucial for an effective immune response since it bridges cellular and humoral immune responses and allows both responses to work synergistically against the same antigens (318). Besides, cDC1 cells also secrete IL-12 (317), which enhances Th1 and NK responses while antagonizing Th2 responses (319). Moreover, cDC1 cells also secrete type-III interferons (317), which strongly polarize $CD4^+$ T-lymphocytes to the Th1 phenotype (320). Noteworthy, cDC1 cells play a central role in the amplitude and effectiveness of immune responses, which makes them great candidates for immunotherapy (321).

Regarding cDC2 cells, these cells activate $CD4^+$ T lymphocytes. They have a huge arsenal of pattern recognition receptors and depending on the microenvironment they can efficiently activate Th1, Th2, Th17, Th22, and follicular Th lymphocytes (317). Therefore, they can modulate a wide range of immune responses. Importantly, cDC2 includes a highly heterogeneous group of cells that need to be further characterized (322). In solid tumors, cDC2 cells have dual roles. They can contribute to antitumoral responses activating Th1, effector Th, and follicular Th lymphocytes. However, they have been poorly studied in the context of tumor immunity compared to cDC1 cells (322). These cDC2 cells can also have pro-tumoral functions. In addition to the activation of Th2 cells, cDC2 cells can further differentiate into regulatory cDCs (DCregs). DCregs suppress effector T lymphocytes to promote a robust antigen-specific tolerance and boost Treg differentiation (323). Nevertheless, this field is very new and much more research is

needed to completely understand the role of cDC2 cells in general and DCregs in particular in the TME.

Noteworthy, the tumor needs to modulate cDCs to tolerogenic phenotypes to escape immune surveillance and promote tumor growth (324). This process is complex and involves multiple mechanisms. Although many authors describe tolerogenic cDCs as a single phenotype, recent research suggests that there are at least two phenotypes (immature or immunosuppressive cDCS) (325). Immature cDCs are present in many tissues under normal conditions and are in charge of maintaining tolerance to harmless antigens. To perform their function, they suppress the activation of effector T cells and promote the expansion of regulatory T cells. Importantly, if they sense 'threat' signals they maturate and promote immune activation instead (325).

Immunosuppressive cDCs, also known as semi-mature cDCs, are not present under normal conditions. They arise in inflammatory microenvironments from alternative activation of immature cDCs and their main function is to limit inflammation (326). They are more efficient in promoting T-lymphocyte anergy and Treg expansion than immature cDCs and also release IL-10 to polarize other immune cells in the microenvironment to an immunosuppressive phenotype (325). Besides, TGF-β and IL-10 enhance the immunosuppressive differentiation of immature cDCs (327). Therefore, the activation of immunosuppressive cDCs generates a feed-forward loop to differentiate immature cDCs into immunosuppressive phenotypes and this loop is enhanced as the tumor progresses due to the accumulation of TGF-β and IL-10 in the TME (328). Importantly, immunosuppressive cDCs cannot maturate to immune-stimulating phenotypes even in the presence of death cells or other 'threat' signals (329,330).

Tumor cells can interact directly with cDCs through Wnt ligands and exosomes. Wnt ligands reprogram cDCs to immunosuppressive phenotypes, including the upregulation of TGF-β and IL-10, as well as promoting the differentiation and proliferation of Tregs (328). Tumor cells can also release exosomes that can increase the upregulation of IL-6 and metalloproteases in cDCs (328). Besides, the tumor can indirectly modulate cDCs by promoting the release of pro-inflammatory mediators, TGF-β, IL-10 and VEGF by other cells in the TME (324,328).

The chronic low-grade pro-inflammatory milieu of the TME causes hyperactivation of STAT3 in cDCs, which prevents their maturation and makes them more responsive to IL-10 and TGF-β (324). Besides, the VEGF released by other cells to promote angiogenesis activates STAT3 and inhibits NF-kB in cDCs, further contributing to their immunosuppressive phenotype (324). Interestingly, STAT3 is negatively regulated by CD45 (331) and the present study shows that there is a decrease in the amount of CD45 present on the surface of cDCs in the tumor microenvironment, which could contribute to the sustained activation of STAT3 and ultimately the development of immunosuppressive cDCs.

Unfortunately, it is unknown if there are changes in the dynamics of immature and immunosuppressive cDCs in the TME, as well as their specific mechanisms of interaction with tumor cells and other immune cells. It may be possible that tolerogenic immature cDCs predominate in early stages of the tumor development and gradually transition to

immunosuppressive cDCs as the tumor progresses. Additionally, it would be interesting to know if immunosuppressive differentiation can occur in both cDCs subpopulations or just in cDC2 cells. Therefore, further research is needed to elucidate the precise molecular and cellular factors that drive the generation and expansion of immunosuppressive cDC subsets in the TME and their interaction with tumor cells and other immune cells.

In CRC, cDC1 cells play a strong anti-tumoral role. In pre-malignant lesions, they prevent the transition from dysplasia to invasive carcinoma by promoting immune surveillance and elimination of neoplastic cells (332). In their absence, tumors progress more rapidly and exhibit reduced infiltration of effector $CD8^+$ T cells, leading to immune evasion (332). Interestingly, cDC1 cells utilize CLEC9A as a means of identifying and engulfing deceased tumor cells in a process known as efferocytosis (333). After that, they cross-present those antigens to $CD8^+$ lymphocytes to promote an antitumoral response and secrete CCL5 to recruit more cDC1 cells. In the presence of Flt3L, this process is enhanced, delaying tumor growth. However, the effect of Flt3L is lost if CLEC9A is KO (333). Besides, the detection of mitochondrial DNA from tumor cells by cDC1 cells triggers the production of type I interferons and enhances cross-presentation, leading to the recruitment and activation of $CD8^+$ T lymphocytes in the TME (334). Therefore, CRC tumors block this mitochondrial DNA sensing pathway in cDC1 cells although the mechanism is not completely understood (334). Unfortunately, most of the papers studying cDCs in CRC do not differentiate between cDC1 and cDC2 cells, which limits our understanding of their specific roles in immune responses.

CRC takes advantage of cDCs' plasticity. It secretes Wnt5a, which triggers the epigenetic inhibition of FOXM1 on cDCs blocking their maturation (335). Since these immature cDCs promote tolerance (314,315), the tumor uses this mechanism to evade immune control. Besides, the immunosuppressive polarization of cDCs also contributes to tumor progression through the release of CXCL1 (336). This CXCL1 enhance stemness features in tumor cells and increase their metastatic potential (336).

In the present study, the number of cDCs increased in the tumor compared to unaffected mucosa and healthy controls. However, since the size of the biopsies was small and cDCs are not very abundant, it was not feasible to subdivide the population of cDCs. Nevertheless, understanding the role of cDCs in the TME and their effect on different populations of T cells it is possible to hypothesize which subpopulation of cDCs may be dominant. Firstly, the number of cDC2 cells is bigger than the one of cDC1 in the colon under normal conditions (337). Secondly, the evasion from cDC1 control is essential in CRC for tumor growth (332–334). Thirdly, in the present study, the increase of $CD4^+$ T lymphocytes is bigger than the one of $CD8^+$. Therefore, it may be possible that the increase is mainly driven by cDC2 cells.

Currently, cDC-based immunotherapy is a very promising field in CRC as it capitalizes on the unique functions and plasticity of cDCs in orchestrating an effective anti-tumoral immune response. The efforts are now focused on two immunotherapies: TLR-agonists and DC vaccines. The activation of TLR signaling pathways in cDCs is a signal of 'threat' and triggers the maturation of cDCs enhancing their antigen presentation capabilities and activating effector T lymphocytes

(338). The use of TLR7 agonists on cDCs in CRC xenograft models has shown promising results in inducing tumor regression and enhancing anti-tumoral immune responses. This effect was achieved through a strong effector T lymphocyte activation and Treg suppression (339). These TLR7 agonists also act on other innate immune cells promoting M1 polarization in macrophages and MDSC suppression (340). Accordingly, clinical trials are going on to test the efficacy and safety of TLR7 agonists in CRC (341). Interestingly, CD45 is an important regulator of TLR signaling in cDCs (221). Notably, in CD45-null mice, cDC1 cells showed impaired maturation and reduced ability to activate Th1 and NK responses (225). The present study reveals a decrease in the amount of CD45 available on the surface of cDCs, which could be a new mechanism to evade cDC control in CRC. This could be used by CRC to become resistant to TLR-agonist immunotherapy and should be taken into account to improve the effectiveness of this type of approach.

In DC vaccines, DC precursors are harvested from a patient, differentiated to DC, induced to maturate with stimulatory cytokine and/or TLR agonists, loaded with tumor antigens, and then reinfused back into the patient (342). This approach had suboptimal results and now there is a huge effort going on to improve the technology. Nowadays, several trials are going on focusing on pDC or cDC2 vaccines in CRC (343). Although cDC1 cells seem more interesting for this type of approach there are some technical difficulties in generating a sufficient number of cDC1 cells for use in DC vaccines (343). Nevertheless, there is currently one clinical trial in melanoma in which they directly inject cDC1 cells into the tumor instead of re-infusing them into the patient, and preliminary results have shown promising efficacy (344). In summary, the use of cDC-based immunotherapy in CRC is really promising but much more basic research and technique development is needed to improve the effectiveness of these approaches.

The role of mast cells in colorectal cancer

Mast cells are present in all vascularized tissues close to blood vessels. Together with DCs and macrophages, they are the sentinel cells of the immune system and one of the first responders to tissular stress (8). However, their main homing location is the gastrointestinal tract, where they play a crucial role in the microenvironment both in homeostasis and disease (345). Mast cells are multifaceted and versatile cells that are never in a 'resting' state. Instead, they are continuously sampling the microenvironment and modulating their response accordingly like a rheostat (346). They store different mediators in different granules and depending on the signal from the microenvironment they selectively release these mediators to modulate the microenvironment. This process is complex and is not completely understood. However, it is known that mast cells include a glycoprotein called serglycin in all their granules and the glycosylation of serglycin changes depending on the granule content (347). This glycoprotein serves as a sort of "barcode" for the granule contents, allowing mast cells to selectively release specific mediators based on the context and signals they receive (348). Unfortunately, it is still unknown which protein acts as the reader of the carbohydrate barcode on serglycin and how the selection process is regulated.

The function of mast cells is not limited to host defense, and they take part in many physiological processes to maintain the homeostasis of the gut mucosa. Firstly, mast cells modulate epithelial activity regulation of water and ion permeability (345), epithelial migration (349), and transport of antigen-antibody complexes across the intestinal barrier (350). Secondly, mast cells also localize close to neural networks in the enteric nervous system and are involved in the regulation of gastrointestinal motility and secretion (351). Enteric neurons regulate the mast cell activation threshold through the release of substance P (352) and calcitonin gene-related peptide (353), which can either promote or inhibit mast cell activation depending on the context (352,353). Conversely, mast cells can also influence the activity of enteric neurons through the release of various mediators, such as histamine and nerve growth factor to modulate peristalsis (354) and intestinal nociception (355). Thirdly, mast cells control the endothelial function. In the short term, mast cells can control blood flow and vascular permeability by releasing vasoactive mediators such as histamine and serotonin (356). In the long term, mast cells can control the size of the microvascular network by promoting the proliferation (108) or apoptosis (357) of endothelial cells. Fourthly, mast cells are master regulators of tissue remodeling including extracellular matrix deposition, fibrosis, and wound healing (358). They can have a direct role through the release of metalloproteases, growth factors, and cytokines that modulate collagen synthesis, and indirectly by promoting fibroblast activity (359). Finally, mast cells also play a crucial role in the regulation of innate and adaptive immune responses within the gut mucosa.

Mast cells can start the canonical innate pathway via pattern recognition receptor activation (TLRs, NLRs, RLRs,...) and the release of cytokines, chemokines, and antimicrobial peptides to initiate an immune response (360). Furthermore, they also have receptors for endogenous danger signals secreted by stressed or injured cells within the gut mucosa, allowing them to sense and respond to tissue damage or infection (361). Noteworthy, since they are already present in the mucosa and they have a huge variety of pre-stored granules that can be rapidly released upon activation, mast cells are among the first responders in immune surveillance and defense in the gastrointestinal tract (362). Mast cells also modulate other innate immune cells. They can promote cDC maturation and migration to lymph nodes secreting prostaglandins, histamine, IL-16, IL-18, TNF-α, and CCL5 (363) or inhibit it through IL-10 (364). Besides, mast cells establish synapses with cDC to transfer them internalized mast cell specific antigens for presentation to T lymphocytes (365). In response to epithelial stress, mast cells release CCL2 and CCL5 (366), which are potent chemoattractants to recruit monocytes and macrophages (367,368). Moreover, mast cells enhance their phagocytic capacity (369) and release TNF-α (370) to promote their polarization toward M1 phenotype (371). Alternatively, mast cells can also promote the polarization of macrophages toward immunomodulatory phenotypes, such as M2 or regulatory macrophages, through the release of TGF-β, IL-10 (364), IL-4 and IL-13 (372).

Finally, mast cells also recruit neutrophils through CXCL1, CXCL2 (373), CXCL8, CXCL10 (362) and TNF-α (370) as well as eosinophils through CCL5, CCL11 (362), IL-5 and MCP-6 (374).

Additionally, mast cells modulate adaptive immune responses. They activate B lymphocytes through CD40L-CD40 interaction and release IL-4 and IL-13 to promote class switch recombination to IgE or TGF-β, IL-5, and IL-6 to promote class switch recombination to IgA (375).

Interestingly, colonic mucosa has a population of B regulatory cells (Bregs) that inhibits Th1 and Th17 responses to prevent chronic colitis and also contributes to IgA secretion to regulate the microbiota in the gut (376). Mast cells control the differentiation and proliferation of Bregs in the colonic mucosa. Their absence significantly reduced the number of Bregs in the colonic mucosa and impairs the resolution of mucosal inflammation (377).

Furthermore, mast cells also establish a bidirectional interaction with T lymphocytes. Mast cells upregulate MHC class-II and costimulatory molecules to activate $CD4^+$ T lymphocytes. Mast cells are well known for inducing the activation and proliferation of Th2 lymphocytes through the release of IL-4 and IL-13 (378). However, they can also promote the activation and proliferation of Th1, Th17 and γδ T lymphocytes although the mechanism is not completely understood (379). Besides, mast cells can also modulate the Th1/Th2/Th17 differentiation indirectly by modulating cDCs (380,381). Mast cells can also activate $CD8^+$ T lymphocytes directly by performing antigen cross-presentation (382) or indirectly by inducing antigen cross-presentation on cDCs (383). Interestingly, mast cells are as efficient as macrophages in direct antigen cross-presentation, but they are less efficient than cDCs in this process (382). Moreover, in an excessive pro-inflammatory microenvironment, mast cells can activate Tregs through the secretion of amphiregulin (384). This rescues Treg activity even under the suppression of Tregs by effector T lymphocytes (384).

On the other hand, mast cells can also suppress T lymphocytes, although there is considerably less information. Mast cells suppress effector T lymphocytes through FasL expression (385), IL-10 (364), and tryptophan deprivation with tryptophan hydroxylase-1 (386). Besides, mast cells suppress Tregs via the secretion of IL-6 and the activation of the OX40-OX40L axis (the latter being expressed in the mast cell) (387). Interestingly, in this OX40-OX40L interaction, Tregs also suppress mast cells although the mechanism is not clear (388). Finally, mast cells can also suppress effector T lymphocytes indirectly by activating Tregs and Bregs, and vice versa.

Importantly, this modulation of T lymphocytes by mast cells is also crucial for the progression from pre-neoplastic lesions to tumor formation. In murine models of hereditary CRC, it was seen that mast cells inhibit Tregs in the mucosa skewing T cell response toward Th17 (389), which generates a chronic inflammation and is essential for the development and progression of CRC (390).

In short, mast cells are crucial for the management of the microenvironment and are continuously adapting to it. Therefore, when analyzing their role in the TME, it is important to consider their dual nature as immune cells and TME managers.

In CRC, mast cells can display pro-tumoral or anti-tumoral roles (391). However, the pro-tumoral functions predominate. This includes mediating angiogenesis, promoting tumor progression and metastasis, and suppressing anti-tumor immune responses (392).

Tumor cells recruit mast cells through CCL15 and stem cell factor (393). After that, mast cells promote tumor growth releasing cytokines like IL-6 and IL-8 (393), that contribute to the

inflammatory milieu and neutrophil recruitment (370). Mast cells can also interact with non-immune cells like fibroblasts. This interaction between mast cells and fibroblasts is poorly understood in CRC but it is known that mast cells can stimulate fibroblasts to produce extracellular matrix components through the release of IL-13, TGF-β, and tryptase, leading to tissue remodeling and potentially facilitating tumor growth and invasion (364,374). Besides, fibroblasts can secrete CCL12 to recruit mast cells (394).

Therefore, mast cells play a critical role in shaping the tumor microenvironment and influencing tumor progression. The present study shows that despite the increase in their number, there are no changes in CD45 and CD203 surface expression. This indicates that signal transduction in mast cells is active both in the tumor and mucosa and that there are no significant changes in the granule release. This agrees with the rheostat model for mast cell interactions with the microenvironment explained before. However, there could be differences in the granule content and the specific molecules released by mast cells in the tumor microenvironment compared to the mucosal microenvironment. This would be an interesting subject for further research to investigate the potential role of mast cell granule content and specific molecules released in the tumor microenvironment, as it could provide valuable insights into the mechanisms by which mast cells contribute to tumor growth and progression in colorectal cancer. Besides, the enigmatic role of CD200 on mast cells and its reduction in the TME compared to the mucosa suggests that CD200 may play a regulatory role in mast cell function and potentially influence tumor progression. Which would be another interesting topic for future study.

CD45 downregulation as a new mechanism to evade immune control

CD45 also known as protein tyrosine phosphatase receptor type C (PTPRC) is a transmembrane protein expressed in all nucleated cells from the hematopoietic lineage. The intracellular part contains a protein tyrosine phosphatase domain (D1) and a second domain (D2) that controls the correct protein folding and substrate attachment (395). The extracellular part includes three fibronectin type III-like repeats, a cysteine-rich domain, and 3 domains known as A, B, and C (exons 4, 5, and 6 respectively) that undergo alternative splicing giving rise to eight isoforms with varying characteristics and functions (396). Therefore, CD45 isoforms are named depending on the ABC domains that they express. For example, CD45RAB express A and B domains but not C, and CD45RO do not express any of the ABC domains. All the extracellular domains of CD45 are heavily glycosylated and this glycosylation changes depending on the cell type, which increases its functional diversity and allows for cell-type specific regulation of signal transduction pathways (397).

In T and B lymphocytes, upon antigen inactivation CD45 removes inhibitory phosphate groups from receptors like T cell receptor (TCR) and B cell receptor (BCR), amplifying downstream signals promoting their activation. This step is so critical that the absence of CD45 causes a severe

combined immunodeficiency (SCID), a syndrome characterized by impaired T and B cell function that is fatal in early childhood if the patient does not receive a bone marrow transplant.

CD45 acts as a regulator of Src family kinases (135), which control several signaling pathways in immune cells including those from receptor-mediated signaling, cytokine signaling, and immune cell proliferation and differentiation (398). This interaction with Src family can be either positive or negative depending on the residue that is dephosphorylated. Besides, CD45 also modulates JAK kinases to regulate the activation of STAT proteins, which play a crucial role in cytokine production, and MyD88 signaling, which is involved in toll-like receptor signaling and innate immune responses (135). The specific effect on each pathway and the selection of the target pathway depends on the isoform of CD45 and the specific context of the immune cell. For example, CD45RO is more efficient than CD45RA in promoting TCR activation signaling, and CD45RO and CD45RB are more efficient in activating STAT3, PKC, and ERK signaling than CD45RA (399). Noteworthy, in T lymphocytes, CD45RA is the most abundant isoform in the naive subset while CD45RO dominates the memory subset (400). This explains why the activation threshold is lower in memory T cells than in the naive subset (399). Furthermore, CD45RABC, previously known as B220 antigen, plays an important role in the differentiation of B (401) and T lymphocytes (402). However, mature T cells express CD45RABC upon activation which enhances Fas signaling and promotes apoptosis to prevent an excessive response (403). In short, CD45 isoforms impact immune cell behavior by modifying activation thresholds, signal transduction, cytokine production, adhesion, memory, and interaction (404). Notably, most of the work on CD45 has been performed on T and B lymphocytes, so there may be other potential mechanisms in innate immune cells that have yet to be fully elucidated.

Interestingly, CD45 activity can be modulated by extracellular ligands. During pregnancy, placenta produces Glycodelin-A, also known as progesterone-associated endometrial protein, which binds to CD45 and inhibits its function. Notably, this inhibition is stronger in CD45RA than in CD45RO, which prevents the activation of naive T cells against fetal antigens while keeping memory T cells active to protect against pathogens (135). Importantly, the effect of Glycodelin-A relies on the alpha 2,6-Sialylation of the glycan chain of CD45 (405). In the absence of this sialylation, the immunomodulatory effect of Glycodelin-A is impaired (406). During cytomegalovirus infection, infected cells express pUL11 from the viral genome. This protein inhibits all CD45 isoforms, leading to impaired T cell activation and immune evasion (135).

Additionally, changes in glycosylation can have a significant impact on CD45 activity. Importantly, CD45 glycosylation is a dynamic process that highly depends on each cell type. In T lymphocytes, different glycosylation patterns of CD45 isoforms have been observed during various stages of cell activation and differentiation (407). These glycosylation changes can affect the recruitment and localization of CD45 to lipid rafts, alter its interactions with other molecules, and modulate its impact on immune cell signaling and activation (408). For example, when there is an addition of N-acetyl galactosamine residues to the glycan chain of CD45 in T lymphocytes it binds the galactose type lectin in the surface of macrophages, which decreases T lymphocyte proliferation, cytokine production, and triggers apoptosis (397). Furthermore, some unidentified changes in glycosylation of CD45 are recognized by galectin-1 and facilitate its binding to CD45, leading to

decreased T cell signaling and potentially promoting cell death (408). Besides, during aging CD45 sialylation decreases, which reduces the responsiveness $CD4^+$ T lymphocytes and contribute to immunosenescence and increased susceptibility to infections in the elderly (135).

Unfortunately, information about CD45 isoforms and glycosylation in innate immune cells is very scarce. Peripheral blood and cultured eosinophils express CD45RO and CD45RB, although the abundance of CD45RO is considerably higher, but never CD45RA (219,409). However, it is still unclear if there are two subpopulations or if both isoforms coexist in the same cells. Importantly, the stimulation of both CD45RO and CD45RB in eosinophils trigger CD11b upregulation and CD62L shedding, which are the main indicators of eosinophil activation (219). After challenge, eosinophils from asthmatic individuals showed increased CD45RO expression compared to healthy controls, suggesting a potential role of CD45RO in asthma pathogenesis. Nevertheless, the functional differences between CD45RO and CD45RB in eosinophils are still unknown and require further investigation.

Neutrophils express CD45RB during their differentiation but switch to CD45RO as they mature (397). Interestingly, resting mature neutrophils also express CD45RA but it is located primarily in intracellular compartments, and it is not exposed on the cell surface (410). Upon activation or inflammatory stimulation, neutrophils translocate CD45RA to the cell surface in a calcium-calmodulin dependent mechanism (411). In neutrophils the activation of both CD45RO and CD45RA trigger the upregulation of CD11b, IL-8, TNF-α and increase their phagocytic capacity (412). CD45RO activation but not CD45RA suppress LCK tyrosine kinase (412), but it is still unclear how this differential activation of CD45 isoforms affects the overall function of neutrophils.

CD45 seem important in DC differentiation. In the absence of CD45, DC precursors show a biased differentiation towards the pDC lineage. However, this pDCs derived from CD45 KO precursors have an impaired ability to produce type I interferon in response to viral stimulation (224). Unfortunately, most of the work on CD45 isoforms was performed in the late 1990's and early 2000's. At that time, the difference between pDCs and cDCs was not well-defined and thus the very few papers that discuss the role of CD45 isoforms in DCs do not differentiate between these two subsets. In general, DCs can express CD45RO and CD45RB (413). CD45RB seems to be important for DCs that migrate to MALTs in the gut, but the exact role of CD45RB is not fully understood (414). Langerhans cells are a subtype of DC only present in skin-associated MALTs with a function like cDCs but with a different ontogeny (415) that only express CD45RO (416). Interestingly, Langerhans cells show considerable diversity in CD45RO glycosylation (416) which may contribute to their specialized functions in the skin although the exact mechanism remains elusive.

In mast cells, CD45 is physically associated to FceRI and modulates it signaling transduction and degranulation through Lyn kinase (417). Noteworthy, in the absence of CD45 in mast cells, IgE mediated degranulation is abolished (418). Besides, CD45 also modulate the signaling of many other receptors, making it indispensable for the effector functions of mast cells (233). In placental and embryonal tissues mast cells express CD45RB and CD45RO (419), while in lung mucosa they express CD45RA, CD45RB, and CD45RO (420). Unfortunately, there is no information about CD45

isoforms in mast cells in normal colon or CRC, but in a case report of a mast cell sarcoma in the colon CD45RB was strongly upregulated (421).

As mentioned before, the study of CD45 isoforms was performed mostly in the 1990's and early 2000's mainly in T and B cells. After that, the field of CD45 isoform research has been relatively quiet, with limited exploration in other immune cell populations such as dendritic cells, eosinophils, neutrophils, and mast cells. Recently, the interest in CD45 has been reignited due to the increased interest in glycobiology and the use of new high throughput techniques in proteomics.

Although there is usually one dominant isoform of CD45 expressed on a single cell, all cells express more than one isoform, which allows for differential glycosylation patterns and functional diversity in immune cell populations. This diversity in CD45 isoform expression and glycosylation patterns enables immune cells to respond dynamically to various stimuli and perform specialized functions. However, the complexity of CD45 glycosylation and isoform expression poses challenges in fully understanding its role in immune cell function that need to be addressed in the upcoming years. Therefore, understanding the expression patterns, regulation, and functional impact of CD45 isoforms in different immune cell populations is crucial for advancing our knowledge of immune cell biology and developing targeted therapies for immune-related disorders including cancer.

Future perspectives

The present study shows that CD45 is downregulated in CRC on eosinophils, neutrophils, cDCs, and pDCs compared to normal mucosa. Given the critical role of CD45 on those cells it is clear that this will impact immune cell function and potentially contribute to the pathogenesis of CRC. Interestingly, the surface abundance of CD45 on eosinophils, pDCs, and cDCs in unaffected mucosa of patients with CRC is significantly reduced compared to the healthy controls. This indicates that the immunological changes triggered by CD45 downregulation in those cells are already present before the tumor arrives. In the case of neutrophils there is a decrease in CD45 in unaffected mucosa, but it is not statistically significant. In the future, it would be interesting to investigate firstly, how the TME modulates the expression of CD45 in innate immune cells. Secondly, how CD45 downregulation affects the immune function of these cells. Thirdly, which are the main CD45 isoforms expressed in innate immune cells in normal mucosa and CRC. Finally, it would be interesting to know if there are glycosylation changes in CD45 isoforms in CRC and how they contribute to immune cell dysfunction.

Conclusions

- The addition of chondroitinase, heparinase I-III, and hyaluronidase to the digestion mix highly improves the quality of the final cell suspension.
- The TME induces changes in the population dynamics of innate immune cells, increasing the number of macrophages, neutrophils, pDCs, cDCs, and mast cells while sharply decreasing the number of eosinophils.
- CD45 is an important and complex modulator of immune cells that is downregulated in neutrophils, eosinophils, pDCs, and cDCs in CRC.
- Some of the changes observed among healthy controls and CRC can be observed also between healthy controls and unaffected mucosa of patients with CRC. Such includes the increased number of neutrophils, the decreased number of eosinophils, the decreased surface abundance of CD44 on mast cells, and the decreased surface abundance of CD45 on eosinophils, pDCs, and cDCs.
- Mast cells reduce the surface expression of CD117, CD44, and CD200 in CRC. Besides, they seem to increase the surface expression of CD123, but we need to increase the sample size to prove it.

Bibliography

1. Hartl FU. Cellular Homeostasis and Aging. Annu Rev Biochem. 2016 Jun 2;85(1):1–4.
2. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2018 Jan 23;9(6):7204–18.
3. Medzhitov R. The spectrum of inflammatory responses. Science (1979). 2021 Nov 26;374(6571):1070–5.
4. Pagán AJ, Ramakrishnan L. The Formation and Function of Granulomas. Annu Rev Immunol. 2018 Apr 26;36(1):639–65.
5. Cronan MR. In the Thick of It: Formation of the Tuberculous Granuloma and Its Effects on Host and Therapeutic Responses. Front Immunol. 2022 Mar 7;13.
6. Scheffer D da L, Latini A. Exercise-induced immune system response: Anti-inflammatory status on peripheral and central organs. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2020 Oct;1866(10):165823.
7. Cerqueira É, Marinho DA, Neiva HP, Lourenço O. Inflammatory Effects of High and Moderate Intensity Exercise—A Systematic Review. Front Physiol. 2020 Jan 9;10.
8. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008 Jul 24;454(7203):428–35.
9. Allaire JM, Crowley SM, Law HT, Chang SY, Ko HJ, Vallance BA. The Intestinal Epithelium: Central Coordinator of Mucosal Immunity. Trends Immunol. 2018 Sep;39(9):677–96.
10. Villalba Lopez D, Kongsbak-Wismann M. Role of IL-22 in homeostasis and diseases of the skin. APMIS. 2022 Jun 6;130(6):314–22.

11. Zaharie RD, Popa C, Schlanger D, Vălean D, Zaharie F. The Role of IL-22 in Wound Healing. Potential Implications in Clinical Practice. Int J Mol Sci. 2022 Mar 28;23(7):3693.
12. Kokubo K, Onodera A, Kiuchi M, Tsuji K, Hirahara K, Nakayama T. Conventional and pathogenic Th2 cells in inflammation, tissue repair, and fibrosis. Front Immunol. 2022 Aug 9;13.
13. Kim SY, Nair MG. Macrophages in wound healing: activation and plasticity. Immunol Cell Biol. 2019 Mar 11;97(3):258–67.
14. Krzyszczyk P, Schloss R, Palmer A, Berthiaume F. The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes. Front Physiol. 2018 May 1;9.
15. Moretti L, Stalfort J, Barker TH, Abebayehu D. The interplay of fibroblasts, the extracellular matrix, and inflammation in scar formation. Journal of Biological Chemistry. 2022 Feb;298(2):101530.
16. McCuaig S, Barras D, Mann EH, Friedrich M, Bullers SJ, Janney A, et al. The Interleukin 22 Pathway Interacts with Mutant KRAS to Promote Poor Prognosis in Colon Cancer. Clinical Cancer Research. 2020 Aug 15;26(16):4313–25.
17. Droeser RA, Iezzi G. IL-22-mediates Cross-talk between Tumor Cells and Immune Cells Associated with Favorable Prognosis in Human Colorectal Cancer. J Cell Immunol. 2021;3(2):118–21.
18. Liu X, Li X, Wei H, Liu Y, Li N. Mast cells in colorectal cancer tumour progression, angiogenesis, and lymphangiogenesis. Front Immunol. 2023 Jul 11;14.
19. Kotsiliti E. Origin of CAFs in colorectal cancer. Nat Rev Gastroenterol Hepatol. 2022 Feb 4;19(2):79–79.
20. Aran D, Lasry A, Zinger A, Biton M, Pikarsky E, Hellman A, et al. Widespread parainflammation in human cancer. Genome Biol. 2016 Dec 8;17(1):145.
21. Boland BB, Rhodes CJ, Grimsby JS. The dynamic plasticity of insulin production in β-cells. Mol Metab. 2017 Sep;6(9):958–73.
22. Krystel-Whittemore M, Dileepan KN, Wood JG. Mast Cell: A Multi-Functional Master Cell. Front Immunol. 2016 Jan 6;6.
23. Kusminski CM, Bickel PE, Scherer PE. Targeting adipose tissue in the treatment of obesity-associated diabetes. Nat Rev Drug Discov. 2016 Sep 3;15(9):639–60.
24. Wernstedt Asterholm I, Tao C, Morley TS, Wang QA, Delgado-Lopez F, Wang ZV, et al. Adipocyte Inflammation Is Essential for Healthy Adipose Tissue Expansion and Remodeling. Cell Metab. 2014 Jul;20(1):103–18.
25. Burhans MS, Hagman DK, Kuzma JN, Schmidt KA, Kratz M. Contribution of Adipose Tissue Inflammation to the Development of Type 2 Diabetes Mellitus. In: Comprehensive Physiology. Wiley; 2018. p. 1–58.
26. Zatterale F, Longo M, Naderi J, Raciti GA, Desiderio A, Miele C, et al. Chronic Adipose Tissue Inflammation Linking Obesity to Insulin Resistance and Type 2 Diabetes. Front Physiol. 2020 Jan 29;10.
27. Tomic D, Shaw JE, Magliano DJ. The burden and risks of emerging complications of diabetes mellitus. Nat Rev Endocrinol. 2022 Sep 6;18(9):525–39.

28. Zhao H, Wu L, Yan G, Chen Y, Zhou M, Wu Y, et al. Inflammation and tumor progression: signaling pathways and targeted intervention. Signal Transduct Target Ther. 2021 Jul 12;6(1):263.
29. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat Rev Endocrinol. 2018 Feb 8;14(2):88–98.
30. Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW, et al. IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. Diabetes Res Clin Pract. 2018 Apr;138:271–81.
31. Galicia-Garcia U, Benito-Vicente A, Jebari S, Larrea-Sebal A, Siddiqi H, Uribe KB, et al. Pathophysiology of Type 2 Diabetes Mellitus. Int J Mol Sci. 2020 Aug 30;21(17):6275.
32. Harding JL, Pavkov ME, Magliano DJ, Shaw JE, Gregg EW. Global trends in diabetes complications: a review of current evidence. Diabetologia. 2019 Jan 31;62(1):3–16.
33. González-Salvatierra S, García-Fontana C, Andújar-Vera F, Grau-Perales AB, Martínez-Heredia L, Avilés-Pérez MD, et al. Osteoglycin as a Potential Biomarker of Mild Kidney Function Impairment in Type 2 Diabetes Patients. J Clin Med. 2021 May 20;10(10):2209.
34. Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. Physiol Rev. 2018 Oct 1;98(4):2133–223.
35. Klip A, McGraw TE, James DE. Thirty sweet years of GLUT4. J Biol Chem. 2019 Jul 26;294(30):11369–81.
36. Soeters MR, Soeters PB. The evolutionary benefit of insulin resistance. Clinical Nutrition. 2012 Dec;31(6):1002–7.
37. Satoh T. Bird evolution by insulin resistance. Trends in Endocrinology & Metabolism. 2021 Oct;32(10):803–13.
38. Watve MG, Yajnik CS. Evolutionary origins of insulin resistance: a behavioral switch hypothesis. BMC Evol Biol. 2007;7(1):61.
39. Colagiuri S, Miller JB. The 'carnivore connection'—evolutionary aspects of insulin resistance. Eur J Clin Nutr. 2002 Mar 1;56(S1):S30–5.
40. Rask-Madsen C, King GL. Vascular Complications of Diabetes: Mechanisms of Injury and Protective Factors. Cell Metab. 2013 Jan;17(1):20–33.
41. Kosiborod M, Gomes MB, Nicolucci A, Pocock S, Rathmann W, Shestakova M V., et al. Vascular complications in patients with type 2 diabetes: prevalence and associated factors in 38 countries (the DISCOVER study program). Cardiovasc Diabetol. 2018 Dec 28;17(1):150.
42. Merkel M, Schmid SM, Iwen KA. Physiologie und klinische Bedeutung von weißem, beigem und braunem Fettgewebe. Internist (Berl). 2019 Feb 8;60(2):115–21.
43. Kajimura S, Spiegelman BM, Seale P. Brown and Beige Fat: Physiological Roles beyond Heat Generation. Cell Metab. 2015 Oct;22(4):546–59.
44. Zoico E, Rubele S, De Caro A, Nori N, Mazzali G, Fantin F, et al. Brown and Beige Adipose Tissue and Aging. Front Endocrinol (Lausanne). 2019 Jun 20;10.
45. Leitner BP, Huang S, Brychta RJ, Duckworth CJ, Baskin AS, McGehee S, et al. Mapping of human brown adipose tissue in lean and obese young men. Proceedings of the National Academy of Sciences. 2017 Aug 8;114(32):8649–54.
46. Machado SA, Pasquarelli-do-Nascimento G, da Silva DS, Farias GR, de Oliveira Santos I, Baptista LB, et al. Browning of the white adipose tissue regulation: new insights into

nutritional and metabolic relevance in health and diseases. Nutr Metab (Lond). 2022 Sep 6;19(1):61.
47. Schena G, Caplan MJ. Everything You Always Wanted to Know about β3-AR * (* But Were Afraid to Ask). Cells. 2019 Apr 16;8(4):357.
48. Martin S. Caveolae, lipid droplets, and adipose tissue biology: pathophysiological aspects. Horm Mol Biol Clin Investig. 2013 Jan 1;15(1).
49. Jeffery E, Wing A, Holtrup B, Sebo Z, Kaplan JL, Saavedra-Peña R, et al. The Adipose Tissue Microenvironment Regulates Depot-Specific Adipogenesis in Obesity. Cell Metab. 2016 Jul;24(1):142–50.
50. Coelho M, Oliveira T, Fernandes R. State of the art paper Biochemistry of adipose tissue: an endocrine organ. Archives of Medical Science. 2013;2:191–200.
51. Wajchenberg BL. Subcutaneous and Visceral Adipose Tissue: Their Relation to the Metabolic Syndrome. Endocr Rev. 2000 Dec 1;21(6):697–738.
52. Recinella L, Orlando G, Ferrante C, Chiavaroli A, Brunetti L, Leone S. Adipokines: New Potential Therapeutic Target for Obesity and Metabolic, Rheumatic, and Cardiovascular Diseases. Front Physiol. 2020 Oct 30;11.
53. Meister BM, Hong SG, Shin J, Rath M, Sayoc J, Park JY. Healthy versus Unhealthy Adipose Tissue Expansion: the Role of Exercise. J Obes Metab Syndr. 2022 Mar 30;31(1):37–50.
54. Horwitz A, Birk R. Adipose Tissue Hyperplasia and Hypertrophy in Common and Syndromic Obesity—The Case of BBS Obesity. Nutrients. 2023 Aug 4;15(15):3445.
55. Marcadenti A, de Abreu-Silva EO. Different adipose tissue depots: Metabolic implications and effects of surgical removal. Endocrinología y Nutrición (English Edition). 2015 Nov;62(9):458–64.
56. Luong Q, Huang J, Lee KY. Deciphering White Adipose Tissue Heterogeneity. Biology (Basel). 2019 Apr 11;8(2):23.
57. Kahn DE, Bergman BC. Keeping It Local in Metabolic Disease: Adipose Tissue Paracrine Signaling and Insulin Resistance. Diabetes. 2022 Apr 1;71(4):599–609.
58. Zhang H, Ding Y, Zeng Q, Wang D, Liu G, Hussain Z, et al. Characteristics of mesenteric adipose tissue attached to different intestinal segments and their roles in immune regulation. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2022 Mar 1;322(3):G310–26.
59. Börgeson E, Boucher J, Hagberg CE. Of mice and men: Pinpointing species differences in adipose tissue biology. Front Cell Dev Biol. 2022 Sep 15;10.
60. O'Connell J, Lynch L, Cawood TJ, Kwasnik A, Nolan N, Geoghegan J, et al. The Relationship of Omental and Subcutaneous Adipocyte Size to Metabolic Disease in Severe Obesity. PLoS One. 2010 Apr 1;5(4):e9997.
61. Jiang L, Sun W, Zhang M, Wang Y, Tian Y, Li P, et al. Omental Adipose Removal Decreases High Blood Pressure in Hypertensive Patients Independent of Body Mass Index. Diabetes Metab Syndr Obes. 2021 Dec;Volume 14:4921–30.
62. Li Y, Mohan H, Lan N, Wu X, Zhou W, Gong J, et al. Mesenteric excision surgery or conservative limited resection in Crohn's disease: study protocol for an international, multicenter, randomized controlled trial. Trials. 2020 Dec 21;21(1):210.
63. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nat Rev Immunol. 2011 Feb 14;11(2):98–107.

64. Lackey DE, Olefsky JM. Regulation of metabolism by the innate immune system. Nat Rev Endocrinol. 2016 Jan 10;12(1):15–28.
65. Lee JJ, Beretvas SN, Freeland-Graves JH. Abdominal Adiposity Distribution in Diabetic/Prediabetic and Nondiabetic Populations: A Meta-Analysis. J Obes. 2014;2014:1–20.
66. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose Tissue Remodeling: Its Role in Energy Metabolism and Metabolic Disorders. Front Endocrinol (Lausanne). 2016 Apr 13;7.
67. Guerrini V, Gennaro ML. Foam Cells: One Size Doesn't Fit All. Trends Immunol. 2019 Dec;40(12):1163–79.
68. Prieur X, Mok CYL, Velagapudi VR, Núñez V, Fuentes L, Montaner D, et al. Differential Lipid Partitioning Between Adipocytes and Tissue Macrophages Modulates Macrophage Lipotoxicity and M2/M1 Polarization in Obese Mice. Diabetes. 2011 Mar 1;60(3):797–809.
69. He F, Huang Y, Song Z, Zhou HJ, Zhang H, Perry RJ, et al. Mitophagy-mediated adipose inflammation contributes to type 2 diabetes with hepatic insulin resistance. Journal of Experimental Medicine. 2021 Mar 1;218(3).
70. Kojta I, Chacińska M, Błachnio-Zabielska A. Obesity, Bioactive Lipids, and Adipose Tissue Inflammation in Insulin Resistance. Nutrients. 2020 May 3;12(5):1305.
71. Li Y, Wong K, Giles A, Jiang J, Lee JW, Adams AC, et al. Hepatic SIRT1 Attenuates Hepatic Steatosis and Controls Energy Balance in Mice by Inducing Fibroblast Growth Factor 21. Gastroenterology. 2014 Feb;146(2):539-549.e7.
72. Perry RJ, Camporez JPG, Kursawe R, Titchenell PM, Zhang D, Perry CJ, et al. Hepatic Acetyl CoA Links Adipose Tissue Inflammation to Hepatic Insulin Resistance and Type 2 Diabetes. Cell. 2015 Feb;160(4):745–58.
73. Cheng R, Ma J xing. Angiogenesis in diabetes and obesity. Rev Endocr Metab Disord. 2015 Mar 10;16(1):67–75.
74. Mukai K, Tsai M, Saito H, Galli SJ. Mast cells as sources of cytokines, chemokines, and growth factors. Immunol Rev. 2018 Mar 12;282(1):121–50.
75. Gilfillan AM, Beaven MA. Regulation of Mast Cell Responses in Health and Disease. Crit Rev Immunol. 2011;31(6):475–530.
76. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol. 2014 Jul 6;14(7):478–94.
77. Tanaka A, Nomura Y, Matsuda A, Ohmori K, Matsuda H. Mast cells function as an alternative modulator of adipogenesis through 15-deoxy-delta-12, 14-prostaglandin J_2. American Journal of Physiology-Cell Physiology. 2011 Dec;301(6):C1360–7.
78. Shi MA, Shi GP. Different Roles of Mast Cells in Obesity and Diabetes: Lessons from Experimental Animals and Humans. Front Immunol. 2012;3.
79. Jung U, Choi MS. Obesity and Its Metabolic Complications: The Role of Adipokines and the Relationship between Obesity, Inflammation, Insulin Resistance, Dyslipidemia and Nonalcoholic Fatty Liver Disease. Int J Mol Sci. 2014 Apr 11;15(4):6184–223.
80. Liu J, Divoux A, Sun J, Zhang J, Clément K, Glickman JN, et al. Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. Nat Med. 2009 Aug 26;15(8):940–5.

81. Gutierrez DA, Muralidhar S, Feyerabend TB, Herzig S, Rodewald HR. Hematopoietic Kit Deficiency, rather than Lack of Mast Cells, Protects Mice from Obesity and Insulin Resistance. Cell Metab. 2015 May;21(5):678–91.
82. Standards of Medical Care in Diabetes—2014. Diabetes Care. 2014 Jan 1;37(Supplement_1):S14–80.
83. Dahlin JS, Hallgren J. Mast cell progenitors: Origin, development and migration to tissues. Mol Immunol. 2015 Jan;63(1):9–17.
84. Bankova LG, Dwyer DF, Liu AY, Austen KF, Gurish MF. Maturation of mast cell progenitors to mucosal mast cells during allergic pulmonary inflammation in mice. Mucosal Immunol. 2015 May;8(3):596–606.
85. Li Z, Liu S, Xu J, Zhang X, Han D, Liu J, et al. Adult Connective Tissue-Resident Mast Cells Originate from Late Erythro-Myeloid Progenitors. Immunity. 2018 Oct;49(4):640-653.e5.
86. Plotkin JD, Elias MG, Fereydouni M, Daniels-Wells TR, Dellinger AL, Penichet ML, et al. Human Mast Cells From Adipose Tissue Target and Induce Apoptosis of Breast Cancer Cells. Front Immunol. 2019 Feb 18;10.
87. Meza-Perez S, Randall TD. Immunological Functions of the Omentum. Trends Immunol. 2017 Jul;38(7):526–36.
88. Bénézech C, Luu NT, Walker JA, Kruglov AA, Loo Y, Nakamura K, et al. Inflammation-induced formation of fat-associated lymphoid clusters. Nat Immunol. 2015 Aug 29;16(8):819–28.
89. Tremaroli V, Bäckhed F. Functional interactions between the gut microbiota and host metabolism. Nature. 2012 Sep 12;489(7415):242–9.
90. Divoux A, Moutel S, Poitou C, Lacasa D, Veyrie N, Aissat A, et al. Mast Cells in Human Adipose Tissue: Link with Morbid Obesity, Inflammatory Status, and Diabetes. J Clin Endocrinol Metab. 2012 Sep 1;97(9):E1677–85.
91. Kumar D, Pandya SK, Varshney S, Shankar K, Rajan S, Srivastava A, et al. Temporal immmunometabolic profiling of adipose tissue in HFD-induced obesity: manifestations of mast cells in fibrosis and senescence. Int J Obes. 2019 Jun 9;43(6):1281–94.
92. Oka T, Kalesnikoff J, Starkl P, Tsai M, Galli SJ. Evidence questioning cromolyn's effectiveness and selectivity as a 'mast cell stabilizer'' in mice.' Laboratory Investigation. 2012 Oct;92(10):1472–82.
93. Forbes EE, Groschwitz K, Abonia JP, Brandt EB, Cohen E, Blanchard C, et al. IL-9– and mast cell–mediated intestinal permeability predisposes to oral antigen hypersensitivity. J Exp Med. 2008 Apr 14;205(4):897–913.
94. Wei Y, Hu Z, Gu W, Liu G, Shi B, Liu E, et al. CD117+CD44+ Stem T Cells Develop in the Thymus and Potently Suppress T-cell Proliferation by Modulating the CTLA-4 Pathway. Stem Cell Res Ther. 2017 Dec 9;8(1):56.
95. Onkanga IO, Hamilton R, Mwinzi PNM, Schneider T, Ondigo BN, Sang H, et al. Expression of CD117 (c-Kit) on Circulating B Cells in Pediatric Schistosomiasis. Infect Immun. 2022 Aug 18;90(8).
96. Dinh HQ, Eggert T, Meyer MA, Zhu YP, Olingy CE, Llewellyn R, et al. Coexpression of CD71 and CD117 Identifies an Early Unipotent Neutrophil Progenitor Population in Human Bone Marrow. Immunity. 2020 Aug;53(2):319-334.e6.

97. Chmelař J, Chatzigeorgiou A, Chung KJ, Prucnal M, Voehringer D, Roers A, et al. No Role for Mast Cells in Obesity-Related Metabolic Dysregulation. Front Immunol. 2016 Nov 24;7.
98. Elieh Ali Komi D, Shafaghat F, Christian M. Crosstalk Between Mast Cells and Adipocytes in Physiologic and Pathologic Conditions. Clin Rev Allergy Immunol. 2020 Jun 25;58(3):388–400.
99. Poglio S, De Toni-Costes F, Arnaud E, Laharrague P, Espinosa E, Casteilla L, et al. Adipose Tissue as a Dedicated Reservoir of Functional Mast Cell Progenitors. Stem Cells. 2010 Nov 1;28(11):2065–72.
100. Cildir G, Pant H, Lopez AF, Tergaonkar V. The transcriptional program, functional heterogeneity, and clinical targeting of mast cells. Journal of Experimental Medicine. 2017 Sep 4;214(9):2491–506.
101. Żelechowska P, Wiktorska M, Różalska S, Stasikowska-Kanicka O, Wągrowska-Danilewicz M, Agier J, et al. Leptin receptor is expressed by tissue mast cells. Immunol Res. 2018 Oct 29;66(5):557–66.
102. Chen C, Khismatullin DB. Oxidized Low-Density Lipoprotein Contributes to Atherogenesis via Co-activation of Macrophages and Mast Cells. PLoS One. 2015 Mar 26;10(3):e0123088.
103. Finlin BS, Zhu B, Confides AL, Westgate PM, Harfmann BD, Dupont-Versteegden EE, et al. Mast Cells Promote Seasonal White Adipose Beiging in Humans. Diabetes. 2017 May 1;66(5):1237–46.
104. Finlin BS, Confides AL, Zhu B, Boulanger MC, Memetimin H, Taylor KW, et al. Adipose Tissue Mast Cells Promote Human Adipose Beiging in Response to Cold. Sci Rep. 2019 Jun 17;9(1):8658.
105. Crewe C, An YA, Scherer PE. The ominous triad of adipose tissue dysfunction: inflammation, fibrosis, and impaired angiogenesis. Journal of Clinical Investigation. 2017 Jan 3;127(1):74–82.
106. Reilly SM, Saltiel AR. Adapting to obesity with adipose tissue inflammation. Nat Rev Endocrinol. 2017 Nov 11;13(11):633–43.
107. Möllerherm H, Branitzki-Heinemann K, Brogden G, Elamin AA, Oehlmann W, Fuhrmann H, et al. Hypoxia Modulates the Response of Mast Cells to Staphylococcus aureus Infection. Front Immunol. 2017 May 11;8.
108. De Souza Junior D, Mazucato V, Santana A, Oliver C, Jamur M. Mast Cells Interact with Endothelial Cells to Accelerate In Vitro Angiogenesis. Int J Mol Sci. 2017 Dec 13;18(12):2674.
109. Mraz M, Haluzik M. The role of adipose tissue immune cells in obesity and low-grade inflammation. Journal of Endocrinology. 2014 Sep;222(3):R113–27.
110. Ishijima Y, Ohmori S, Ohneda K. Mast cell deficiency results in the accumulation of preadipocytes in adipose tissue in both obese and non-obese mice. FEBS Open Bio. 2014 Jan 28;4(1):18–24.
111. Samuel VT, Shulman GI. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. Journal of Clinical Investigation. 2016 Jan 4;126(1):12–22.

112. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure. Eur Heart J. 2016 Jul 14;37(27):2129–200.
113. Valencak TG, Osterrieder A, Schulz TJ. Sex matters: The effects of biological sex on adipose tissue biology and energy metabolism. Redox Biol. 2017 Aug;12:806–13.
114. Bel Lassen P, Charlotte F, Liu Y, Bedossa P, Le Naour G, Tordjman J, et al. The FAT Score, a Fibrosis Score of Adipose Tissue: Predicting Weight-Loss Outcome After Gastric Bypass. J Clin Endocrinol Metab. 2017 Jul 1;102(7):2443–53.
115. Divoux A, Tordjman J, Lacasa D, Veyrie N, Hugol D, Aissat A, et al. Fibrosis in Human Adipose Tissue: Composition, Distribution, and Link With Lipid Metabolism and Fat Mass Loss. Diabetes. 2010 Nov 1;59(11):2817–25.
116. Muir LA, Neeley CK, Meyer KA, Baker NA, Brosius AM, Washabaugh AR, et al. Adipose tissue fibrosis, hypertrophy, and hyperplasia: Correlations with diabetes in human obesity. Obesity. 2016 Mar 25;24(3):597–605.
117. Sun K, Tordjman J, Clément K, Scherer PE. Fibrosis and Adipose Tissue Dysfunction. Cell Metab. 2013 Oct;18(4):470–7.
118. Amiot L, Vu N, Drenou B, Scrofani M, Chalin A, Devisme C, et al. The anti-fibrotic role of mast cells in the liver is mediated by HLA-G and interaction with hepatic stellate cells. Cytokine. 2019 May;117:50–8.
119. García-Rubio J, León J, Redruello-Romero A, Pavón E, Cozar A, Tamayo F, et al. Cytometric analysis of adipose tissue reveals increments of adipocyte progenitor cells after weight loss induced by bariatric surgery. Sci Rep. 2018 Oct 12;8(1):15203.
120. Goldstein N, Kezerle Y, Gepner Y, Haim Y, Pecht T, Gazit R, et al. Higher Mast Cell Accumulation in Human Adipose Tissues Defines Clinically Favorable Obesity Sub-Phenotypes. Cells. 2020 Jun 20;9(6):1508.
121. Rönnberg E, Melo FR, Pejler G. Mast Cell Proteoglycans. Journal of Histochemistry & Cytochemistry. 2012 Dec 16;60(12):950–62.
122. Kornstädt L, Pierre S, Weigert A, Ebersberger S, Schäufele TJ, Kolbinger A, et al. Bacterial and Fungal Toll-Like Receptor Activation Elicits Type I IFN Responses in Mast Cells. Front Immunol. 2021 Feb 12;11.
123. Migalovich-Sheikhet H, Friedman S, Mankuta D, Levi-Schaffer F. Novel Identified Receptors on Mast Cells. Front Immunol. 2012;3.
124. Murakami T, Suzuki K, Niyonsaba F, Tada H, Reich J, Tamura H, et al. MrgX2-mediated internalization of LL-37 and degranulation of human LAD2 mast cells. Mol Med Rep. 2018 Oct 2;
125. Bulfone-Paus S, Nilsson G, Draber P, Blank U, Levi-Schaffer F. Positive and Negative Signals in Mast Cell Activation. Trends Immunol. 2017 Sep;38(9):657–67.
126. Valent P, Cerny-Reiterer S, Herrmann H, Mirkina I, George TI, Sotlar K, et al. Phenotypic heterogeneity, novel diagnostic markers, and target expression profiles in normal and neoplastic human mast cells. Best Pract Res Clin Haematol. 2010 Sep;23(3):369–78.
127. Cop N, Decuyper I, Faber M, Sabato V, Bridts C, Hagendorens M, et al. Phenotypic and functional characterization of *in vitro* cultured human mast cells. Cytometry B Clin Cytom. 2017 Sep;92(5):348–54.

128. O'Gorman MR, Corrochano V. Rapid whole-blood flow cytometry assay for diagnosis of chronic granulomatous disease. Clinical Diagnostic Laboratory Immunology. 1995 Mar;2(2):227–32.
129. Baris HE, Ogulur I, Akcam B, Kiykim A, Karagoz D, Saraymen B, et al. Diagnostic Modalities Based on Flow Cytometry for Chronic Granulomatous Disease: A Multicenter Study in a Well-Defined Cohort. J Allergy Clin Immunol Pract. 2020 Nov;8(10):3525-3534.e1.
130. Björkbacka H, Yao Mattisson I, Wigren M, Melander O, Fredrikson GN, Bengtsson E, et al. Plasma stem cell factor levels are associated with risk of cardiovascular disease and death. J Intern Med. 2017 Dec;282(6):508–21.
131. Bouzina H, Rådegran G. Low plasma stem cell factor combined with high transforming growth factor-α identifies high-risk patients in pulmonary arterial hypertension. ERJ Open Res. 2018 Oct 12;4(4):00035–2018.
132. Sibilano R, Frossi B, Pucillo CE. Mast cell activation: A complex interplay of positive and negative signaling pathways. Eur J Immunol. 2014 Sep;44(9):2558–66.
133. Asai K, Kitaura J, Kawakami Y, Yamagata N, Tsai M, Carbone DP, et al. Regulation of Mast Cell Survival by IgE. Immunity. 2001 Jun;14(6):791–800.
134. Bax HJ, Keeble AH, Gould HJ. Cytokinergic IgE Action in Mast Cell Activation. Front Immunol. 2012;3.
135. Rheinländer A, Schraven B, Bommhardt U. CD45 in human physiology and clinical medicine. Immunol Lett. 2018 Apr;196:22–32.
136. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018 Nov;68(6):394–424.
137. Rawla P, Sunkara T, Barsouk A. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. Gastroenterology Review. 2019;14(2):89–103.
138. Fleming M, Ravula S, Tatishchev SF, Wang HL. Colorectal carcinoma: Pathologic aspects. J Gastrointest Oncol. 2012 Sep;3(3):153–73.
139. Taieb J, André T, Auclin E. Refining adjuvant therapy for non-metastatic colon cancer, new standards and perspectives. Cancer Treat Rev. 2019 May;75:1–11.
140. Li Y, Zhao L, Güngör C, Tan F, Zhou Z, Li C, et al. The main contributor to the upswing of survival in locally advanced colorectal cancer: an analysis of the SEER database. Therap Adv Gastroenterol. 2019 Jan 18;12:175628481986215.
141. Riihimäki M, Hemminki A, Sundquist J, Hemminki K. Patterns of metastasis in colon and rectal cancer. Sci Rep. 2016 Jul 15;6(1):29765.
142. Rex DK, Boland RC, Dominitz JA, Giardiello FM, Johnson DA, Kaltenbach T, et al. Colorectal Cancer Screening: Recommendations for Physicians and Patients from the U.S. Multi-Society Task Force on Colorectal Cancer. American Journal of Gastroenterology. 2017 Jul;112(7):1016–30.
143. Shapiro JA, Soman A V., Berkowitz Z, Fedewa SA, Sabatino SA, de Moor JS, et al. Screening for Colorectal Cancer in the United States: Correlates and Time Trends by Type of Test. Cancer Epidemiology, Biomarkers & Prevention. 2021 Aug 1;30(8):1554–65.
144. Forster S, Radpour R. Molecular Immunotherapy: Promising Approach to Treat Metastatic Colorectal Cancer by Targeting Resistant Cancer Cells or Cancer Stem Cells. Front Oncol. 2020 Nov 9;10.

145. Zhuang Y, Wang H, Jiang D, Li Y, Feng L, Tian C, et al. Multi gene mutation signatures in colorectal cancer patients: predict for the diagnosis, pathological classification, staging and prognosis. BMC Cancer. 2021 Dec 9;21(1):380.
146. Etienne-Grimaldi MC, Formento JL, Francoual M, François E, Formento P, Renée N, et al. K-Ras Mutations and Treatment Outcome in Colorectal Cancer Patients Receiving Exclusive Fluoropyrimidine Therapy. Clinical Cancer Research. 2008 Aug 1;14(15):4830–5.
147. Lasry A, Zinger A, Ben-Neriah Y. Inflammatory networks underlying colorectal cancer. Nat Immunol. 2016 Mar 16;17(3):230–40.
148. Wu D, Wu P, Huang Q, Liu Y, Ye J, Huang J. Interleukin-17: A Promoter in Colorectal Cancer Progression. Clin Dev Immunol. 2013;2013:1–7.
149. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. Nature. 2012 Nov 7;491(7423):254–8.
150. Porter RJ, Arends MJ, Churchhouse AMD, Din S. Inflammatory Bowel Disease-Associated Colorectal Cancer: Translational Risks from Mechanisms to Medicines. J Crohns Colitis. 2021 Dec 18;15(12):2131–41.
151. Dienstmann R, Vermeulen L, Guinney J, Kopetz S, Tejpar S, Tabernero J. Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. Nat Rev Cancer. 2017 Feb 4;17(2):79–92.
152. Thanki K, Nicholls ME, Gajjar A, Senagore AJ, Qiu S, Szabo C, et al. Consensus Molecular Subtypes of Colorectal Cancer and their Clinical Implications. Int Biol Biomed J. 2017;3(3):105–11.
153. Roelands J, Kuppen P, Vermeulen L, Maccalli C, Decock J, Wang E, et al. Immunogenomic Classification of Colorectal Cancer and Therapeutic Implications. Int J Mol Sci. 2017 Oct 24;18(10):2229.
154. Pino MS, Chung DC. The Chromosomal Instability Pathway in Colon Cancer. Gastroenterology. 2010 May;138(6):2059–72.
155. Berg KCG, Sveen A, Høland M, Alagaratnam S, Berg M, Danielsen SA, et al. Gene expression profiles of CMS2-epithelial/canonical colorectal cancers are largely driven by DNA copy number gains. Oncogene. 2019 Aug 15;38(33):6109–22.
156. Dai Z, Peng X, Guo Y, Shen X, Ding W, Fu J, et al. Metabolic pathway-based molecular subtyping of colon cancer reveals clinical immunotherapy potential and prognosis. J Cancer Res Clin Oncol. 2023 Jun 22;149(6):2393–416.
157. Strating E, Wassenaar E, Verhagen M, Rauwerdink P, van Schelven S, de Hingh I, et al. Fibroblast activation protein identifies Consensus Molecular Subtype 4 in colorectal cancer and allows its detection by 68Ga-FAPI-PET imaging. Br J Cancer. 2022 Jul 1;127(1):145–55.
158. Peters NA, Constantinides A, Ubink I, van Kuik J, Bloemendal HJ, van Dodewaard JM, et al. Consensus molecular subtype 4 (CMS4)-targeted therapy in primary colon cancer: A proof-of-concept study. Front Oncol. 2022 Sep 6;12.
159. Walter RJ, Sonnentag SJ, Orian-Rousseau V, Munoz-Sagredo L. Plasticity in Colorectal Cancer: Why Cancer Cells Differentiate. Cancers (Basel). 2021 Feb 22;13(4).
160. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar;144(5):646–74.

161. Giakoustidis A, Mudan S, Hagemann T. Tumour Microenvironment: Overview with an Emphasis on the Colorectal Liver Metastasis Pathway. Cancer Microenvironment. 2015 Dec 3;8(3):177–86.
162. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013 Nov 7;19(11):1423–37.
163. Vessoni AT, Filippi-Chiela EC, Lenz G, Batista LFZ. Tumor propagating cells: drivers of tumor plasticity, heterogeneity, and recurrence. Oncogene. 2020 Mar 5;39(10):2055–68.
164. MacLeod AS, Kwock JT. Inflammation in Wound Repair: Role and Function of Inflammation in Wound Repair. In: Wound Healing. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2017. p. 177–94.
165. Lasry A, Ben-Neriah Y. Senescence-associated inflammatory responses: aging and cancer perspectives. Trends Immunol. 2015 Apr;36(4):217–28.
166. Pribluda A, Elyada E, Wiener Z, Hamza H, Goldstein RE, Biton M, et al. A Senescence-Inflammatory Switch from Cancer-Inhibitory to Cancer-Promoting Mechanism. Cancer Cell. 2013 Aug;24(2):242–56.
167. van den Boogaard WMC, Komninos DSJ, Vermeij WP. Chemotherapy Side-Effects: Not All DNA Damage Is Equal. Cancers (Basel). 2022 Jan 26;14(3):627.
168. Cuccu A, Francescangeli F, De Angelis ML, Bruselles A, Giuliani A, Zeuner A. Analysis of Dormancy-Associated Transcriptional Networks Reveals a Shared Quiescence Signature in Lung and Colorectal Cancer. Int J Mol Sci. 2022 Aug 30;23(17):9869.
169. Batlle E, Clevers H. Cancer stem cells revisited. Nat Med. 2017 Oct 1;23(10):1124–34.
170. Takasugi M, Yoshida Y, Hara E, Ohtani N. The role of cellular senescence and SASP in tumour microenvironment. FEBS J. 2023 Mar 20;290(5):1348–61.
171. Pedrosa L, Esposito F, Thomson TM, Maurel J. The Tumor Microenvironment in Colorectal Cancer Therapy. Cancers (Basel). 2019 Aug 14;11(8):1172.
172. Sugawara T, Miya F, Ishikawa T, Lysenko A, Nishino J, Kamatani T, et al. Immune subtypes and neoantigen-related immune evasion in advanced colorectal cancer. iScience. 2022 Feb;25(2):103740.
173. Picard E, Verschoor CP, Ma GW, Pawelec G. Relationships Between Immune Landscapes, Genetic Subtypes and Responses to Immunotherapy in Colorectal Cancer. Front Immunol. 2020 Mar 6;11.
174. Soldevilla B, Carretero-Puche C, Gomez-Lopez G, Al-Shahrour F, Riesco MC, Gil-Calderon B, et al. The correlation between immune subtypes and consensus molecular subtypes in colorectal cancer identifies novel tumour microenvironment profiles, with prognostic and therapeutic implications. Eur J Cancer. 2019 Dec;123:118–29.
175. Bae JM, Yoo SY, Kim JH, Kang GH. Immune landscape and biomarkers for immuno-oncology in colorectal cancers. J Pathol Transl Med. 2020 Sep 15;54(5):351–60.
176. Zhang L, Zhao Y, Dai Y, Cheng JN, Gong Z, Feng Y, et al. Immune Landscape of Colorectal Cancer Tumor Microenvironment from Different Primary Tumor Location. Front Immunol. 2018 Jul 10;9.
177. Khaliq AM, Erdogan C, Kurt Z, Turgut SS, Grunvald MW, Rand T, et al. Refining colorectal cancer classification and clinical stratification through a single-cell atlas. Genome Biol. 2022 Dec 11;23(1):113.

178. Wu X, Yan H, Qiu M, Qu X, Wang J, Xu S, et al. Comprehensive characterization of tumor microenvironment in colorectal cancer via molecular analysis. Elife. 2023 Jun 2;12.
179. Purcell R V., Schmeier S, Lau YC, Pearson JF, Frizelle FA. Molecular subtyping improves prognostication of Stage 2 colorectal cancer. BMC Cancer. 2019 Dec 27;19(1):1155.
180. ten Hoorn S, de Back TR, Sommeijer DW, Vermeulen L. Clinical Value of Consensus Molecular Subtypes in Colorectal Cancer: A Systematic Review and Meta-Analysis. JNCI: Journal of the National Cancer Institute. 2022 Apr 11;114(4):503–16.
181. Hu F, Wang J, Zhang M, Wang S, Zhao L, Yang H, et al. Comprehensive Analysis of Subtype-Specific Molecular Characteristics of Colon Cancer: Specific Genes, Driver Genes, Signaling Pathways, and Immunotherapy Responses. Front Cell Dev Biol. 2021 Nov 29;9.
182. Sagaert X, Vanstapel A, Verbeek S. Tumor Heterogeneity in Colorectal Cancer: What Do We Know So Far? Pathobiology. 2018;85(1–2):72–84.
183. Karpinski P, Rossowska J, Sasiadek MM. Immunological landscape of consensus clusters in colorectal cancer. Oncotarget. 2017 Dec 1;8(62):105299–311.
184. He X, Xu C. Immune checkpoint signaling and cancer immunotherapy. Cell Res. 2020 Aug 28;30(8):660–9.
185. Weng J, Li S, Zhu Z, Liu Q, Zhang R, Yang Y, et al. Exploring immunotherapy in colorectal cancer. J Hematol Oncol. 2022 Dec 16;15(1):95.
186. Golshani G, Zhang Y. Advances in immunotherapy for colorectal cancer: a review. Therap Adv Gastroenterol. 2020 Jan 1;13:175628482091752.
187. Mulet-Margalef N, Linares J, Badia-Ramentol J, Jimeno M, Sanz Monte C, Manzano Mozo JL, et al. Challenges and Therapeutic Opportunities in the dMMR/MSI-H Colorectal Cancer Landscape. Cancers (Basel). 2023 Feb 6;15(4):1022.
188. Wang S, Guan X, Ma M, Zhuang M, Ma T, Liu Z, et al. Reconsidering the prognostic significance of tumour deposit count in the TNM staging system for colorectal cancer. Sci Rep. 2020 Jan 9;10(1):89.
189. Pei JP, Zhang CD, Fu X, Ba Y, Yue S, Zhao ZM, et al. A Novel TNM Classification for Colorectal Cancers based on the Metro-ticket Paradigm. J Cancer. 2021;12(11):3299–306.
190. Galon J, Mlecnik B, Bindea G, Angell HK, Berger A, Lagorce C, et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. J Pathol. 2014 Jan 10;232(2):199–209.
191. Bruni D, Angell HK, Galon J. The immune contexture and Immunoscore in cancer prognosis and therapeutic efficacy. Nat Rev Cancer. 2020 Nov 4;20(11):662–80.
192. Hermitte F. Biomarkers immune monitoring technology primer: Immunoscore® Colon. J Immunother Cancer. 2016 Dec 20;4(1):57.
193. Galon J, Lanzi A. Immunoscore and its introduction in clinical practice. The Quarterly Journal of Nuclear Medicine and Molecular Imaging. 2020 May;64(2).
194. Marliot F, Pagès F, Galon J. Usefulness and robustness of Immunoscore for personalized management of cancer patients. Oncoimmunology. 2020 Jan 1;9(1).
195. Zheng Z, Wieder T, Mauerer B, Schäfer L, Kesselring R, Braumüller H. T Cells in Colorectal Cancer: Unravelling the Function of Different T Cell Subsets in the Tumor Microenvironment. Int J Mol Sci. 2023 Jul 19;24(14):11673.
196. Inamura K. Colorectal Cancers: An Update on Their Molecular Pathology. Cancers (Basel). 2018 Jan 20;10(1):26.

197. Magaki S, Hojat SA, Wei B, So A, Yong WH. An Introduction to the Performance of Immunohistochemistry. In 2019. p. 289–98.
198. Tan WCC, Nerurkar SN, Cai HY, Ng HHM, Wu D, Wee YTF, et al. Overview of multiplex immunohistochemistry/immunofluorescence techniques in the era of cancer immunotherapy. Cancer Commun (Lond). 2020 Apr;40(4):135–53.
199. Vandereyken K, Sifrim A, Thienpont B, Voet T. Methods and applications for single-cell and spatial multi-omics. Nat Rev Genet. 2023 Aug 2;24(8):494–515.
200. Jaye DL, Bray RA, Gebel HM, Harris WAC, Waller EK. Translational Applications of Flow Cytometry in Clinical Practice. The Journal of Immunology. 2012 May 15;188(10):4715–9.
201. Betters DM. Use of Flow Cytometry in Clinical Practice. J Adv Pract Oncol. 2015;6(5):435–40.
202. Terho P, Lassila O. Novel method for cell debris removal in the flow cytometric cell cycle analysis using carboxy-fluorescein diacetate succinimidyl ester. Cytometry Part A. 2006 Jun;69A(6):552–4.
203. Drescher H, Weiskirchen S, Weiskirchen R. Flow Cytometry: A Blessing and a Curse. Biomedicines. 2021 Nov 4;9(11).
204. Galon J, Angell HK, Bedognetti D, Marincola FM. The Continuum of Cancer Immunosurveillance: Prognostic, Predictive, and Mechanistic Signatures. Immunity. 2013 Jul;39(1):11–26.
205. Ijsselsteijn ME, Brouwer TP, Abdulrahman Z, Reidy E, Ramalheiro A, Heeren AM, et al. Cancer immunophenotyping by seven-colour multispectral imaging without tyramide signal amplification. J Pathol Clin Res. 2019 Jan;5(1):3–11.
206. Noy R, Pollard JW. Tumor-Associated Macrophages: From Mechanisms to Therapy. Immunity. 2014 Jul;41(1):49–61.
207. Tosolini M, Kirilovsky A, Mlecnik B, Fredriksen T, Mauger S, Bindea G, et al. Clinical Impact of Different Classes of Infiltrating T Cytotoxic and Helper Cells (Th1, Th2, Treg, Th17) in Patients with Colorectal Cancer. Cancer Res. 2011 Feb 15;71(4):1263–71.
208. Lugli A, Karamitopoulou E, Panayiotides I, Karakitsos P, Rallis G, Peros G, et al. CD8+ lymphocytes/ tumour-budding index: an independent prognostic factor representing a 'pro-/anti-tumour' approach to tumour host interaction in colorectal cancer. Br J Cancer. 2009 Oct 15;101(8):1382–92.
209. Grasso CS, Giannakis M, Wells DK, Hamada T, Mu XJ, Quist M, et al. Genetic Mechanisms of Immune Evasion in Colorectal Cancer. Cancer Discov. 2018 Jun 1;8(6):730–49.
210. Mlecnik B, Tosolini M, Kirilovsky A, Berger A, Bindea G, Meatchi T, et al. Histopathologic-Based Prognostic Factors of Colorectal Cancers Are Associated With the State of the Local Immune Reaction. Journal of Clinical Oncology. 2011 Feb 20;29(6):610–8.
211. Haram A, Boland MR, Kelly ME, Bolger JC, Waldron RM, Kerin MJ. The prognostic value of neutrophil-to-lymphocyte ratio in colorectal cancer: A systematic review. J Surg Oncol. 2017 Mar;115(4):470–9.
212. Walsh SR, Cook EJ, Goulder F, Justin TA, Keeling NJ. Neutrophil-lymphocyte ratio as a prognostic factor in colorectal cancer. J Surg Oncol. 2005 Sep 1;91(3):181–4.
213. Galdiero MR, Bianchi P, Grizzi F, Di Caro G, Basso G, Ponzetta A, et al. Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer. Int J Cancer. 2016 Jul 15;139(2):446–56.

214. Governa V, Trella E, Mele V, Tornillo L, Amicarella F, Cremonesi E, et al. The Interplay Between Neutrophils and CD8+ T Cells Improves Survival in Human Colorectal Cancer. Clinical Cancer Research. 2017 Jul 15;23(14):3847–58.
215. Zhu JW, Doan K, Park J, Chau AH, Zhang H, Lowell CA, et al. Receptor-like Tyrosine Phosphatases CD45 and CD148 Have Distinct Functions in Chemoattractant-Mediated Neutrophil Migration and Response to S. aureus. Immunity. 2011 Nov;35(5):757–69.
216. Travers J, Rothenberg ME. Eosinophils in mucosal immune responses. Mucosal Immunol. 2015 May;8(3):464–75.
217. Arnold IC, Artola-Boran M, Gurtner A, Bertram K, Bauer M, Frangez Z, et al. The GM-CSF–IRF5 signaling axis in eosinophils promotes antitumor immunity through activation of type 1 T cell responses. Journal of Experimental Medicine. 2020 Dec 7;217(12).
218. Carretero R, Sektioglu IM, Garbi N, Salgado OC, Beckhove P, Hämmerling GJ. Eosinophils orchestrate cancer rejection by normalizing tumor vessels and enhancing infiltration of CD8+ T cells. Nat Immunol. 2015 Jun 27;16(6):609–17.
219. Matsumoto K, Bochner B, Wakiguchi H, Kurashige T. Altered Expression of CD11b and CD62L after Cross-Linking of CD45 Isoforms on Human Eosinophils. Int Arch Allergy Immunol. 1998;117(Suppl. 1):34–9.
220. Zhou B, Lawrence T, Liang Y. The Role of Plasmacytoid Dendritic Cells in Cancers. Front Immunol. 2021 Oct 19;12.
221. Saunders AE, Johnson P. Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45. Cell Signal. 2010 Mar;22(3):339–48.
222. Abe K, Nguyen KP, Fine SD, Mo JH, Shen C, Shenouda S, et al. Conventional dendritic cells regulate the outcome of colonic inflammation independently of T cells. Proceedings of the National Academy of Sciences. 2007 Oct 23;104(43):17022–7.
223. Legitimo A, Consolini R, Failli A, Orsini G, Spisni R. Dendritic cell defects in the colorectal cancer. Hum Vaccin Immunother. 2014 Nov 2;10(11):3224–35.
224. Montoya M, Dawes R, Reid D, Lee LN, Piercy J, Borrow P, et al. CD45 is required for type I IFN production by dendritic cells. Eur J Immunol. 2006 Aug;36(8):2150–8.
225. Cross JL, Kott K, Miletić T, Johnson P. CD45 Regulates TLR-Induced Proinflammatory Cytokine and IFN-β Secretion in Dendritic Cells. The Journal of Immunology. 2008 Jun 15;180(12):8020–9.
226. Yu Y, Blokhuis B, Garssen J, Redegeld F. A Transcriptomic Insight into the Impact of Colon Cancer Cells on Mast Cells. Int J Mol Sci. 2019 Apr 4;20(7):1689.
227. Song F, Zhang Y, Chen Q, Bi D, Yang M, Lu L, et al. Mast cells inhibit colorectal cancer development by inducing ER stress through secreting Cystatin C. Oncogene. 2023 Jan 12;42(3):209–23.
228. Sammarco G, Gallo G, Vescio G, Picciariello A, De Paola G, Trompetto M, et al. Mast Cells, microRNAs and Others: The Role of Translational Research on Colorectal Cancer in the Forthcoming Era of Precision Medicine. J Clin Med. 2020 Sep 3;9(9):2852.
229. Nielsen HJ, Hansen U, Christensen IJ, Reimert CM, Brünner N, Moesgaard F. Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue. J Pathol. 1999 Dec;189(4):487–95.

230. Suzuki S, Ichikawa Y, Nakagawa K, Kumamoto T, Mori R, Matsuyama R, et al. High infiltration of mast cells positive to tryptase predicts worse outcome following resection of colorectal liver metastases. BMC Cancer. 2015 Dec 4;15(1):840.
231. Wu X, Zou Y, He X, Yuan R, Chen Y, Lan N, et al. Tumor-Infiltrating Mast Cells in Colorectal Cancer as a Poor Prognostic Factor. Int J Surg Pathol. 2013 Apr 30;21(2):111–20.
232. Felce JH, Sezgin E, Wane M, Brouwer H, Dustin ML, Eggeling C, et al. CD45 exclusion– and cross-linking–based receptor signaling together broaden FcεRI reactivity. Sci Signal. 2018 Dec 18;11(561).
233. Grochowy G, Hermiston ML, Kuhny M, Weiss A, Huber M. Requirement for CD45 in fine-tuning mast cell responses mediated by different ligand–receptor systems. Cell Signal. 2009 Aug;21(8):1277–86.
234. Chisholm KM, Merker JD, Gotlib JR, Gitana G, Lefterova M, Zehnder JL, et al. Mast Cells in Systemic Mastocytosis Have Distinctly Brighter CD45 Expression by Flow Cytometry. Am J Clin Pathol. 2015 Apr 1;143(4):527–34.
235. Nagata Y, Suzuki R. FcεRI: A Master Regulator of Mast Cell Functions. Cells. 2022 Feb 11;11(4):622.
236. Metcalfe DD, Pawankar R, Ackerman SJ, Akin C, Clayton F, Falcone FH, et al. Biomarkers of the involvement of mast cells, basophils and eosinophils in asthma and allergic diseases. World Allergy Organization Journal. 2016;9:7.
237. Valent P, Cerny-Reiterer S, Herrmann H, Mirkina I, George TI, Sotlar K, et al. Phenotypic heterogeneity, novel diagnostic markers, and target expression profiles in normal and neoplastic human mast cells. Best Pract Res Clin Haematol. 2010 Sep;23(3):369–78.
238. Tsai M, Valent P, Galli SJ. KIT as a master regulator of the mast cell lineage. Journal of Allergy and Clinical Immunology. 2022 Jun;149(6):1845–54.
239. Caslin HL, Kiwanuka KN, Haque TT, Taruselli MT, MacKnight HP, Paranjape A, et al. Controlling Mast Cell Activation and Homeostasis: Work Influenced by Bill Paul That Continues Today. Front Immunol. 2018 Apr 26;9.
240. Sullivan KM, Jiang X, Guha P, Lausted C, Carter JA, Hsu C, et al. Blockade of interleukin 10 potentiates antitumour immune function in human colorectal cancer liver metastases. Gut. 2023 Feb;72(2):325–37.
241. Mroczko B, Szmitkowski M, WereszczyńSka-Siemiątkowska U, Okulczyk B. Stem Cell Factor (SCF) and Interleukin 3 (IL-3) in the Sera of Patients with Colorectal Cancer. Dig Dis Sci. 2005 Jun 1;50(6):1019–24.
242. Hallgren J, Gurish MF. Mast Cell Progenitor Trafficking and Maturation. In 2011. p. 14–28.
243. Broughton SE, Hercus TR, Nero TL, Kan WL, Barry EF, Dottore M, et al. A dual role for the N-terminal domain of the IL-3 receptor in cell signalling. Nat Commun. 2018 Jan 26;9(1):386.
244. Caslin HL, Kiwanuka KN, Haque TT, Taruselli MT, MacKnight HP, Paranjape A, et al. Controlling Mast Cell Activation and Homeostasis: Work Influenced by Bill Paul That Continues Today. Front Immunol. 2018 Apr 26;9.
245. Senbanjo LT, Chellaiah MA. CD44: A Multifunctional Cell Surface Adhesion Receptor Is a Regulator of Progression and Metastasis of Cancer Cells. Front Cell Dev Biol. 2017 Mar 7;5.

246. Tanaka S. Targeting CD44 in mast cell regulation. Expert Opin Ther Targets. 2010 Jan 9;14(1):31–43.
247. Li ZL, Wang ZJ, Wei GH, Yong Y, Wang XW. Changes in extracellular matrix in different stages of colorectal cancer and their effects on proliferation of cancer cells. World J Gastrointest Oncol. 2020 Mar 15;12(3):267–75.
248. Shao A, Owens DM. The immunoregulatory protein CD200 as a potentially lucrative yet elusive target for cancer therapy. Oncotarget. 2023 Feb 4;14(1):96–103.
249. Liu JQ, Hu A, Zhu J, Yu J, Talebian F, Bai XF. CD200-CD200R Pathway in the Regulation of Tumor Immune Microenvironment and Immunotherapy. In 2020. p. 155–65.
250. Wong KK, Zhu F, Khatri I, Huo Q, Spaner DE, Gorczynski RM. Characterization of CD200 Ectodomain Shedding. PLoS One. 2016 Apr 25;11(4):e0152073.
251. Schupp J, Christians A, Zimmer N, Gleue L, Jonuleit H, Helm M, et al. In-Depth Immune-Oncology Studies of the Tumor Microenvironment in a Humanized Melanoma Mouse Model. Int J Mol Sci. 2021 Jan 20;22(3):1011.
252. Lopez-Perez D, Redruello-Romero A, Garcia-Rubio J, Arana C, Garcia-Escudero LA, Tamayo F, et al. In Patients With Obesity, the Number of Adipose Tissue Mast Cells Is Significantly Lower in Subjects With Type 2 Diabetes. Front Immunol. 2021 May 21;12.
253. Wijnands AM, de Jong ME, Lutgens MWMD, Hoentjen F, Elias SG, Oldenburg B. Prognostic Factors for Advanced Colorectal Neoplasia in Inflammatory Bowel Disease: Systematic Review and Meta-analysis. Gastroenterology. 2021 Apr;160(5):1584–98.
254. Zhang C, Zhang J, Zhang Y, Song Z, Bian J, Yi H, et al. Identifying neutrophil-associated subtypes in ulcerative colitis and confirming neutrophils promote colitis-associated colorectal cancer. Front Immunol. 2023 Feb 10;14.
255. Ghimire J, Iftikhar R, Penrose HM, Snarski P, Ruiz E, Savkovic SD. FOXO3 Deficiency in Neutrophils Drives Colonic Inflammation and Tumorigenesis. Int J Mol Sci. 2023 Jun 4;24(11):9730.
256. Cai Q, Kim M, Harada A, Idowu MO, Sundaresan G, Zweit J, et al. Alpha-1 Antitrypsin Inhibits Tumorigenesis and Progression of Colitis-Associated Colon Cancer through Suppression of Inflammatory Neutrophil-Activated Serine Proteases and IGFBP-3 Proteolysis. Int J Mol Sci. 2022 Nov 8;23(22):13737.
257. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell. 2011 Mar;144(5):646–74.
258. Hwang WL, Lan HY, Cheng WC, Huang SC, Yang MH. Tumor stem-like cell-derived exosomal RNAs prime neutrophils for facilitating tumorigenesis of colon cancer. J Hematol Oncol. 2019 Dec 25;12(1):10.
259. Bui TM, Butin-Israeli V, Wiesolek HL, Zhou M, Rehring JF, Wiesmüller L, et al. Neutrophils Alter DNA Repair Landscape to Impact Survival and Shape Distinct Therapeutic Phenotypes of Colorectal Cancer. Gastroenterology. 2021 Jul;161(1):225-238.e15.
260. Qin F, Liu X, Chen J, Huang S, Wei W, Zou Y, et al. Anti-TGF-β attenuates tumor growth via polarization of tumor associated neutrophils towards an anti-tumor phenotype in colorectal cancer. J Cancer. 2020;11(9):2580–92.
261. Giese MA, Hind LE, Huttenlocher A. Neutrophil plasticity in the tumor microenvironment. Blood. 2019 May 16;133(20):2159–67.

262. Jackstadt R, van Hooff SR, Leach JD, Cortes-Lavaud X, Lohuis JO, Ridgway RA, et al. Epithelial NOTCH Signaling Rewires the Tumor Microenvironment of Colorectal Cancer to Drive Poor-Prognosis Subtypes and Metastasis. Cancer Cell. 2019 Sep;36(3):319-336.e7.
263. Germann M, Zangger N, Sauvain M, Sempoux C, Bowler AD, Wirapati P, et al. Neutrophils suppress tumor-infiltrating T cells in colon cancer via matrix metalloproteinase-mediated activation of <scp>TGF</scp> β. EMBO Mol Med. 2020 Jan 9;12(1).
264. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. 2018 Feb 9;18(2):134–47.
265. Stehr AM, Wang G, Demmler R, Stemmler MP, Krug J, Tripal P, et al. Neutrophil extracellular traps drive epithelial–mesenchymal transition of human colon cancer. J Pathol. 2022 Apr 15;256(4):455–67.
266. Rayes RF, Vourtzoumis P, Bou Rjeily M, Seth R, Bourdeau F, Giannias B, et al. Neutrophil Extracellular Trap–Associated CEACAM1 as a Putative Therapeutic Target to Prevent Metastatic Progression of Colon Carcinoma. The Journal of Immunology. 2020 Apr 15;204(8):2285–94.
267. Mizuno R, Kawada K, Itatani Y, Ogawa R, Kiyasu Y, Sakai Y. The Role of Tumor-Associated Neutrophils in Colorectal Cancer. Int J Mol Sci. 2019 Jan 27;20(3):529.
268. Wågsäter D, Löfgren S, Hugander A, Dienus O, Dimberg J. Analysis of single nucleotide polymorphism in the promoter and protein expression of the chemokine Eotaxin-1 in colorectal cancer patients. World J Surg Oncol. 2007 Dec 31;5(1):84.
269. Bonecchi R, Graham GJ. Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response. Front Immunol. 2016 Jun 10;7.
270. Hughes CE, Nibbs RJB. A guide to chemokines and their receptors. FEBS J. 2018 Aug 24;285(16):2944–71.
271. Zajkowska M, Mroczko B. Eotaxins and Their Receptor in Colorectal Cancer—A Literature Review. Cancers (Basel). 2020 May 28;12(6):1383.
272. Davis BP, Rothenberg ME. Eosinophils and Cancer. Cancer Immunol Res. 2014 Jan 1;2(1):1–8.
273. Reichman H, Karo-Atar D, Munitz A. Emerging Roles for Eosinophils in the Tumor Microenvironment. Trends Cancer. 2016 Nov;2(11):664–75.
274. Hodzic Z, Schill EM, Bolock AM, Good M. IL-33 and the intestine: The good, the bad, and the inflammatory. Cytokine. 2017 Dec;100:1–10.
275. Bui TM, Wiesolek HL, Sumagin R. ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis. J Leukoc Biol. 2020 Sep 1;108(3):787–99.
276. Zaki MH, Vogel P, Body-Malapel M, Lamkanfi M, Kanneganti TD. IL-18 Production Downstream of the Nlrp3 Inflammasome Confers Protection against Colorectal Tumor Formation. The Journal of Immunology. 2010 Oct 15;185(8):4912–20.
277. Gatault S, Delbeke M, Driss V, Sarazin A, Dendooven A, Kahn JE, et al. IL-18 Is Involved in Eosinophil-Mediated Tumoricidal Activity against a Colon Carcinoma Cell Line by Upregulating LFA-1 and ICAM-1. The Journal of Immunology. 2015 Sep 1;195(5):2483–92.
278. Legrand F, Driss V, Delbeke M, Loiseau S, Hermann E, Dombrowicz D, et al. Human Eosinophils Exert TNF-α and Granzyme A-Mediated Tumoricidal Activity toward Colon Carcinoma Cells. The Journal of Immunology. 2010 Dec 15;185(12):7443–51.

279. Reichman H, Itan M, Rozenberg P, Yarmolovski T, Brazowski E, Varol C, et al. Activated Eosinophils Exert Antitumorigenic Activities in Colorectal Cancer. Cancer Immunol Res. 2019 Mar 1;7(3):388–400.
280. Mamtimin M, Pinarci A, Han C, Braun A, Anders HJ, Gudermann T, et al. Extracellular DNA Traps: Origin, Function and Implications for Anti-Cancer Therapies. Front Oncol. 2022 Apr 27;12.
281. Karki R, Man SM, Kanneganti TD. Inflammasomes and Cancer. Cancer Immunol Res. 2017 Feb 1;5(2):94–9.
282. Andreone S, Spadaro F, Buccione C, Mancini J, Tinari A, Sestili P, et al. IL-33 Promotes CD11b/CD18-Mediated Adhesion of Eosinophils to Cancer Cells and Synapse-Polarized Degranulation Leading to Tumor Cell Killing. Cancers (Basel). 2019 Oct 26;11(11):1664.
283. Kienzl M, Hasenoehrl C, Valadez-Cosmes P, Maitz K, Sarsembayeva A, Sturm E, et al. IL-33 reduces tumor growth in models of colorectal cancer with the help of eosinophils. Oncoimmunology. 2020 Jan 1;9(1).
284. Germic N, Fettrelet T, Stojkov D, Hosseini A, Horn MP, Karaulov A, et al. The Release Kinetics of Eosinophil Peroxidase and Mitochondrial DNA Is Different in Association with Eosinophil Extracellular Trap Formation. Cells. 2021 Feb 3;10(2):306.
285. Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E, et al. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nat Med. 2008 Sep 10;14(9):949–53.
286. Ueki S, Melo RCN, Ghiran I, Spencer LA, Dvorak AM, Weller PF. Eosinophil extracellular DNA trap cell death mediates lytic release of free secretion-competent eosinophil granules in humans. Blood. 2013 Mar 14;121(11):2074–83.
287. Acharya KR, Ackerman SJ. Eosinophil Granule Proteins: Form and Function. Journal of Biological Chemistry. 2014 Jun;289(25):17406–15.
288. Gaur P, Zaffran I, George T, Rahimli Alekberli F, Ben-Zimra M, Levi-Schaffer F. The regulatory role of eosinophils in viral, bacterial, and fungal infections. Clin Exp Immunol. 2022 Jul 22;209(1):72–82.
289. Siemińska I, Poljańska E, Baran J. Granulocytes and Cells of Granulocyte Origin—The Relevant Players in Colorectal Cancer. Int J Mol Sci. 2021 Apr 6;22(7):3801.
290. Qin M, Wang L, Li F, Yang M, Song L, Tian F, et al. Oxidized LDL activated eosinophil polarize macrophage phenotype from M2 to M1 through activation of CD36 scavenger receptor. Atherosclerosis. 2017 Aug;263:82–91.
291. Huang Y, Goel S, Duda DG, Fukumura D, Jain RK. Vascular Normalization as an Emerging Strategy to Enhance Cancer Immunotherapy. Cancer Res. 2013 May 15;73(10):2943–8.
292. Cho H, Lim SJ, Won KY, Bae GE, Kim GY, Min JW, et al. Eosinophils in Colorectal Neoplasms Associated with Expression of CCL11 and CCL24. J Pathol Transl Med. 2016 Jan 15;50(1):45–51.
293. Abtahi S, Davani F, Mojtahedi Z, Hosseini S, Bananzadeh A, Ghaderi A. Dual association of serum interleukin-10 levels with colorectal cancer. J Cancer Res Ther. 2017;13(2):252.
294. Pryczynicz A, Guzińska-Ustymowicz K, Kemona A. Fas/FasL expression in colorectal cancer. An immunohistochemical study. Folia Histochem Cytobiol. 2010 Nov 13;48(3).
295. Ilmarinen P, Moilanen E, Kankaanranta H. Regulation of Spontaneous Eosinophil Apoptosis—A Neglected Area of Importance. J Cell Death. 2014 Jan 10;7:JCD.S13588.

296. Qiu Y, Nguyen KD, Odegaard JI, Cui X, Tian X, Locksley RM, et al. Eosinophils and Type 2 Cytokine Signaling in Macrophages Orchestrate Development of Functional Beige Fat. Cell. 2014 Jun;157(6):1292–308.
297. Liu L, Zhang Y, Zheng X, Jin L, Xiang N, Zhang M, et al. Eosinophils attenuate arthritis by inducing M2 macrophage polarization via inhibiting the IκB/P38 MAPK signaling pathway. Biochem Biophys Res Commun. 2019 Jan;508(3):894–901.
298. Harbaum L, Pollheimer MJ, Kornprat P, Lindtner RA, Bokemeyer C, Langner C. Peritumoral eosinophils predict recurrence in colorectal cancer. Modern Pathology. 2015 Mar;28(3):403–13.
299. Koucký V, Bouček J, Fialová A. Immunology of Plasmacytoid Dendritic Cells in Solid Tumors: A Brief Review. Cancers (Basel). 2019 Apr 3;11(4):470.
300. Kießler M, Plesca I, Sommer U, Wehner R, Wilczkowski F, Müller L, et al. Tumor-infiltrating plasmacytoid dendritic cells are associated with survival in human colon cancer. J Immunother Cancer. 2021 Mar;9(3):e001813.
301. Hong EH, Cho J, Ahn J hee, Kwon BE, Kweon MN, Seo SU, et al. Plasmacytoid dendritic cells regulate colitis-associated tumorigenesis by controlling myeloid-derived suppressor cell infiltration. Cancer Lett. 2020 Nov;493:102–12.
302. Bourquin C, Hotz C, Noerenberg D, Voelkl A, Heidegger S, Roetzer LC, et al. Systemic Cancer Therapy with a Small Molecule Agonist of Toll-like Receptor 7 Can Be Improved by Circumventing TLR Tolerance. Cancer Res. 2011 Aug 1;71(15):5123–33.
303. Triozzi PL, Aldrich W, Ponnazhagan S. Inhibition and promotion of tumor growth with adeno-associated virus carcinoembryonic antigen vaccine and Toll-like receptor agonists. Cancer Gene Ther. 2011 Dec 26;18(12):850–8.
304. Vermi W, Soncini M, Melocchi L, Sozzani S, Facchetti F. Plasmacytoid dendritic cells and cancer. J Leukoc Biol. 2011 Jul 5;90(4):681–90.
305. Terra M, Oberkampf M, Fayolle C, Rosenbaum P, Guillerey C, Dadaglio G, et al. Tumor-Derived TGFβ Alters the Ability of Plasmacytoid Dendritic Cells to Respond to Innate Immune Signaling. Cancer Res. 2018 Jun 1;78(11):3014–26.
306. Jahrsdörfer B, Vollmer A, Blackwell SE, Maier J, Sontheimer K, Beyer T, et al. Granzyme B produced by human plasmacytoid dendritic cells suppresses T-cell expansion. Blood. 2010 Feb 11;115(6):1156–65.
307. Castillo-González R, Valle-Noguera A, Gomez-Sánchez MJ, Xia P, Cruz-Adalia A. Innate lymphoid cells type 3 in cancer. Front Immunol. 2022 Oct 13;13.
308. Aspord C, Leccia MT, Charles J, Plumas J. Plasmacytoid Dendritic Cells Support Melanoma Progression by Promoting Th2 and Regulatory Immunity through OX40L and ICOSL. Cancer Immunol Res. 2013 Dec 1;1(6):402–15.
309. Camisaschi C, De Filippo A, Beretta V, Vergani B, Villa A, Vergani E, et al. Alternative Activation of Human Plasmacytoid DCs In Vitro and in Melanoma Lesions: Involvement of LAG-3. Journal of Investigative Dermatology. 2014 Jul;134(7):1893–902.
310. Ghirelli C, Reyal F, Jeanmougin M, Zollinger R, Sirven P, Michea P, et al. Breast Cancer Cell–Derived GM-CSF Licenses Regulatory Th2 Induction by Plasmacytoid Predendritic Cells in Aggressive Disease Subtypes. Cancer Res. 2015 Jul 15;75(14):2775–87.
311. Dey M, Chang AL, Miska J, Wainwright DA, Ahmed AU, Balyasnikova I V., et al. Dendritic Cell–Based Vaccines that Utilize Myeloid Rather than Plasmacytoid Cells Offer a Superior

Survival Advantage in Malignant Glioma. The Journal of Immunology. 2015 Jul 1;195(1):367–76.
312. Sawant A, Hensel JA, Chanda D, Harris BA, Siegal GP, Maheshwari A, et al. Depletion of Plasmacytoid Dendritic Cells Inhibits Tumor Growth and Prevents Bone Metastasis of Breast Cancer Cells. The Journal of Immunology. 2012 Nov 1;189(9):4258–65.
313. Bernhard S, Hug S, Stratmann AEP, Erber M, Vidoni L, Knapp CL, et al. Interleukin 8 Elicits Rapid Physiological Changes in Neutrophils That Are Altered by Inflammatory Conditions. J Innate Immun. 2021;13(4):225–41.
314. Wculek SK, Cueto FJ, Mujal AM, Melero I, Krummel MF, Sancho D. Dendritic cells in cancer immunology and immunotherapy. Nat Rev Immunol. 2020 Jan 29;20(1):7–24.
315. Gardner A, de Mingo Pulido Á, Ruffell B. Dendritic Cells and Their Role in Immunotherapy. Front Immunol. 2020 May 21;11.
316. Cabeza-Cabrerizo M, Cardoso A, Minutti CM, Pereira da Costa M, Reis e Sousa C. Dendritic Cells Revisited. Annu Rev Immunol. 2021 Apr 26;39(1):131–66.
317. Collin M, Bigley V. Human dendritic cell subsets: an update. Immunology. 2018 May;154(1):3–20.
318. Embgenbroich M, Burgdorf S. Current Concepts of Antigen Cross-Presentation. Front Immunol. 2018 Jul 16;9.
319. Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, et al. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. Journal of Experimental Medicine. 1993 Apr 1;177(4):1199–204.
320. Manivasagam S, Klein RS. Type III Interferons: Emerging Roles in Autoimmunity. Front Immunol. 2021 Nov 26;12.
321. Zhang S, Chopin M, Nutt SL. Type 1 conventional dendritic cells: ontogeny, function, and emerging roles in cancer immunotherapy. Trends Immunol. 2021 Dec;42(12):1113–27.
322. Saito Y, Komori S, Kotani T, Murata Y, Matozaki T. The Role of Type-2 Conventional Dendritic Cells in the Regulation of Tumor Immunity. Cancers (Basel). 2022 Apr 13;14(8):1976.
323. Ness S, Lin S, Gordon JR. Regulatory Dendritic Cells, T Cell Tolerance, and Dendritic Cell Therapy for Immunologic Disease. Front Immunol. 2021 Mar 10;12.
324. Lin A, Schildknecht A, Nguyen LT, Ohashi PS. Dendritic cells integrate signals from the tumor microenvironment to modulate immunity and tumor growth. Immunol Lett. 2010 Jan;127(2):77–84.
325. Hasegawa H, Matsumoto T. Mechanisms of Tolerance Induction by Dendritic Cells In Vivo. Front Immunol. 2018 Feb 26;9.
326. Geisel J, Kahl F, Müller M, Wagner H, Kirschning CJ, Autenrieth IB, et al. IL-6 and Maturation Govern TLR2 and TLR4 Induced TLR Agonist Tolerance and Cross-Tolerance in Dendritic Cells. The Journal of Immunology. 2007 Nov 1;179(9):5811–8.
327. Bourque J, Hawiger D. Life and death of tolerogenic dendritic cells. Trends Immunol. 2023 Feb;44(2):110–8.
328. DeVito NC, Plebanek MP, Theivanthiran B, Hanks BA. Role of Tumor-Mediated Dendritic Cell Tolerization in Immune Evasion. Front Immunol. 2019 Dec 10;10.

329. Devi K. SP, Anandasabapathy N. The origin of DCs and capacity for immunologic tolerance in central and peripheral tissues. Semin Immunopathol. 2017 Feb 25;39(2):137–52.
330. García-González P, Ubilla-Olguín G, Catalán D, Schinnerling K, Aguillón JC. Tolerogenic dendritic cells for reprogramming of lymphocyte responses in autoimmune diseases. Autoimmun Rev. 2016 Nov;15(11):1071–80.
331. Kumar V, Cheng P, Condamine T, Mony S, Languino LR, McCaffrey JC, et al. CD45 Phosphatase Inhibits STAT3 Transcription Factor Activity in Myeloid Cells and Promotes Tumor-Associated Macrophage Differentiation. Immunity. 2016 Feb;44(2):303–15.
332. Wang L, Yu S, Chan ER, Chen KY, Liu C, Che D, et al. Notch-Regulated Dendritic Cells Restrain Inflammation-Associated Colorectal Carcinogenesis. Cancer Immunol Res. 2021 Mar 1;9(3):348–61.
333. Cueto FJ, del Fresno C, Brandi P, Combes AJ, Hernández-García E, Sánchez-Paulete AR, et al. DNGR-1 limits Flt3L-mediated antitumor immunity by restraining tumor-infiltrating type I conventional dendritic cells. J Immunother Cancer. 2021 May 12;9(5):e002054.
334. Xu MM, Pu Y, Han D, Shi Y, Cao X, Liang H, et al. Dendritic Cells but Not Macrophages Sense Tumor Mitochondrial DNA for Cross-priming through Signal Regulatory Protein α Signaling. Immunity. 2017 Aug;47(2):363-373.e5.
335. Zhou Z, Chen H, Xie R, Wang H, Li S, Xu Q, et al. Epigenetically modulated <scp>FOXM</scp> 1 suppresses dendritic cell maturation in pancreatic cancer and colon cancer. Mol Oncol. 2019 Apr 15;13(4):873–93.
336. Hsu YL, Chen YJ, Chang WA, Jian SF, Fan HL, Wang JY, et al. Interaction between Tumor-Associated Dendritic Cells and Colon Cancer Cells Contributes to Tumor Progression via CXCL1. Int J Mol Sci. 2018 Aug 16;19(8):2427.
337. Guilliams M, Dutertre CA, Scott CL, McGovern N, Sichien D, Chakarov S, et al. Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. Immunity. 2016 Sep;45(3):669–84.
338. Hou B, Reizis B, DeFranco AL. Toll-like Receptors Activate Innate and Adaptive Immunity by using Dendritic Cell-Intrinsic and -Extrinsic Mechanisms. Immunity. 2008 Aug;29(2):272–82.
339. Wang C, Zhou Q, Wang X, Wu X, Chen X, Li J, et al. The TLR7 agonist induces tumor regression both by promoting CD4+T cells proliferation and by reversing T regulatory cell-mediated suppression via dendritic cells. Oncotarget. 2015 Jan 30;6(3):1779–89.
340. Liu Z, Xie Y, Xiong Y, Liu S, Qiu C, Zhu Z, et al. TLR 7/8 agonist reverses oxaliplatin resistance in colorectal cancer via directing the myeloid-derived suppressor cells to tumoricidal M1-macrophages. Cancer Lett. 2020 Jan;469:173–85.
341. Miller A, Le T, Holland J, Weitzman R, Wu T. 1167 APR003, an oral liver- and GI-targeted TLR7 agonist, elicits a robust type I interferon response in advanced colorectal cancer patients. In: Regular and Young Investigator Award Abstracts. BMJ Publishing Group Ltd; 2022. p. A1208–A1208.
342. Garg AD, Coulie PG, Van den Eynde BJ, Agostinis P. Integrating Next-Generation Dendritic Cell Vaccines into the Current Cancer Immunotherapy Landscape. Trends Immunol. 2017 Aug;38(8):577–93.

343. Calmeiro J, Carrascal MA, Tavares AR, Ferreira DA, Gomes C, Falcão A, et al. Dendritic Cell Vaccines for Cancer Immunotherapy: The Role of Human Conventional Type 1 Dendritic Cells. Pharmaceutics. 2020 Feb 15;12(2):158.
344. Schwarze JK, Tijtgat J, Awada G, Cras L, Vasaturo A, Bagnall C, et al. Intratumoral administration of CD1c (BDCA-1) $^{+}$ and CD141 (BDCA-3) $^{+}$ myeloid dendritic cells in combination with talimogene laherparepvec in immune checkpoint blockade refractory advanced melanoma patients: a phase I clinical trial. J Immunother Cancer. 2022 Sep 16;10(9):e005141.
345. Albert-Bayo M, Paracuellos I, González-Castro AM, Rodríguez-Urrutia A, Rodríguez-Lagunas MJ, Alonso-Cotoner C, et al. Intestinal Mucosal Mast Cells: Key Modulators of Barrier Function and Homeostasis. Cells. 2019 Feb 8;8(2):135.
346. Frossi B, Mion F, Tripodo C, Colombo MP, Pucillo CE. Rheostatic Functions of Mast Cells in the Control of Innate and Adaptive Immune Responses. Trends Immunol. 2017 Sep;38(9):648–56.
347. Rönnberg E, Pejler G. Serglycin: The Master of the Mast Cell. In 2012. p. 201–17.
348. Mulloy B, Lever R, Page CP. Mast cell glycosaminoglycans. Glycoconj J. 2017 Jun 30;34(3):351–61.
349. Groschwitz KR, Ahrens R, Osterfeld H, Gurish MF, Han X, Åbrink M, et al. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. Proceedings of the National Academy of Sciences. 2009 Dec 29;106(52):22381–6.
350. Tu YH, Oluwole C, Struiksma S, Perdue MH, Yang PC. Mast cells modulate transport of CD23/IgE/antigen complex across human intestinal epithelial barrier. N Am J Med Sci. 2009 Jun;1(1):16–24.
351. Yashiro T, Ogata H, Zaidi SF, Lee J, Hayashi S, Yamamoto T, et al. Pathophysiological Roles of Neuro-Immune Interactions between Enteric Neurons and Mucosal Mast Cells in the Gut of Food Allergy Mice. Cells. 2021 Jun 23;10(7):1586.
352. Wang J, Hauer-Jensen M. Neuroimmune interactions: potential target for mitigating or treating intestinal radiation injury. Br J Radiol. 2007 Sep;80(special_issue_1):S41–8.
353. Kim JH, Yamamoto T, Lee J, Yashiro T, Hamada T, Hayashi S, et al. CGRP, a neurotransmitter of enteric sensory neurons, contributes to the development of food allergy due to the augmentation of microtubule reorganization in mucosal mast cells. Biomed Res. 2014;35(4):285–93.
354. De Winter BY, van den Wijngaard RM, de Jonge WJ. Intestinal mast cells in gut inflammation and motility disturbances. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2012 Jan;1822(1):66–73.
355. van Diest SA, Stanisor OI, Boeckxstaens GE, de Jonge WJ, van den Wijngaard RM. Relevance of mast cell–nerve interactions in intestinal nociception. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2012 Jan;1822(1):74–84.
356. Kunder CA, St John AL, Abraham SN. Mast cell modulation of the vascular and lymphatic endothelium. Blood. 2011 Nov 17;118(20):5383–93.
357. Lätti S, Leskinen M, Shiota N, Wang Y, Kovanen PT, Lindstedt KA. Mast cell-mediated apoptosis of endothelial cells in vitro: A paracrine mechanism involving TNF-α-mediated down-regulation of bcl-2 expression. J Cell Physiol. 2003 Apr;195(1):130–8.

358. Ng MF. The role of mast cells in wound healing. Int Wound J. 2010 Feb;7(1):55–61.
359. Komi DEA, Khomtchouk K, Santa Maria PL. A Review of the Contribution of Mast Cells in Wound Healing: Involved Molecular and Cellular Mechanisms. Clin Rev Allergy Immunol. 2020 Jun 7;58(3):298–312.
360. Dietrich N, Rohde M, Geffers R, Kröger A, Hauser H, Weiss S, et al. Mast cells elicit proinflammatory but not type I interferon responses upon activation of TLRs by bacteria. Proceedings of the National Academy of Sciences. 2010 May 11;107(19):8748–53.
361. St. John AL, Abraham SN. Innate Immunity and Its Regulation by Mast Cells. The Journal of Immunology. 2013 May 1;190(9):4458–63.
362. Cardamone C, Parente R, Feo G De, Triggiani M. Mast cells as effector cells of innate immunity and regulators of adaptive immunity. Immunol Lett. 2016 Oct;178:10–4.
363. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol. 2005 Feb 20;6(2):135–42.
364. Komi DEA, Redegeld FA. Role of Mast Cells in Shaping the Tumor Microenvironment. Clin Rev Allergy Immunol. 2020 Jun 29;58(3):313–25.
365. Carroll-Portillo A, Cannon JL, te Riet J, Holmes A, Kawakami Y, Kawakami T, et al. Mast cells and dendritic cells form synapses that facilitate antigen transfer for T cell activation. Journal of Cell Biology. 2015 Aug 31;210(5):851–64.
366. Bawazeer MA, Theoharides TC. IL-33 stimulates human mast cell release of CCL5 and CCL2 via MAPK and NF-κB, inhibited by methoxyluteolin. Eur J Pharmacol. 2019 Dec;865:172760.
367. Keophiphath M, Rouault C, Divoux A, Clément K, Lacasa D. CCL5 Promotes Macrophage Recruitment and Survival in Human Adipose Tissue. Arterioscler Thromb Vasc Biol. 2010 Jan;30(1):39–45.
368. Xu R, Li Y, Yan H, Zhang E, Huang X, Chen Q, et al. CCL2 promotes macrophages-associated chemoresistance via MCPIP1 dual catalytic activities in multiple myeloma. Cell Death Dis. 2019 Oct 14;10(10):781.
369. De Zuani M, Paolicelli G, Zelante T, Renga G, Romani L, Arzese A, et al. Mast Cells Respond to Candida albicans Infections and Modulate Macrophages Phagocytosis of the Fungus. Front Immunol. 2018 Nov 30;9.
370. Dudeck J, Kotrba J, Immler R, Hoffmann A, Voss M, Alexaki VI, et al. Directional mast cell degranulation of tumor necrosis factor into blood vessels primes neutrophil extravasation. Immunity. 2021 Mar;54(3):468-483.e5.
371. Yao Y, Xu XH, Jin L. Macrophage Polarization in Physiological and Pathological Pregnancy. Front Immunol. 2019 Apr 15;10.
372. Galli SJ, Gaudenzio N, Tsai M. Mast Cells in Inflammation and Disease: Recent Progress and Ongoing Concerns. Annu Rev Immunol. 2020 Apr 26;38(1):49–77.
373. De Filippo K, Dudeck A, Hasenberg M, Nye E, van Rooijen N, Hartmann K, et al. Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. Blood. 2013 Jun 13;121(24):4930–7.
374. Elieh Ali Komi D, Wöhrl S, Bielory L. Mast Cell Biology at Molecular Level: a Comprehensive Review. Clin Rev Allergy Immunol. 2020 Jun 12;58(3):342–65.
375. Merluzzi S, Betto E, Ceccaroni AA, Magris R, Giunta M, Mion F. Mast cells, basophils and B cell connection network. Mol Immunol. 2015 Jan;63(1):94–103.

376. Melcher C, Yu J, Duong VHH, Westphal K, Helmi Siasi Farimany N, Shaverskyi A, et al. B cell-mediated regulatory mechanisms control tumor-promoting intestinal inflammation. Cell Rep. 2022 Jul;40(2):111051.
377. Mion F, D'Incà F, Danelli L, Toffoletto B, Guarnotta C, Frossi B, et al. Mast Cells Control the Expansion and Differentiation of IL-10–Competent B Cells. The Journal of Immunology. 2014 Nov 1;193(9):4568–79.
378. Mekori YA, Hershko AY, Frossi B, Mion F, Pucillo CE. Integrating innate and adaptive immune cells: Mast cells as crossroads between regulatory and effector B and T cells. Eur J Pharmacol. 2016 May;778:84–9.
379. Katsoulis-Dimitriou K, Kotrba J, Voss M, Dudeck J, Dudeck A. Mast Cell Functions Linking Innate Sensing to Adaptive Immunity. Cells. 2020 Nov 25;9(12):2538.
380. Dudeck A, Suender CA, Kostka SL, von Stebut E, Maurer M. Mast cells promote Th1 and Th17 responses by modulating dendritic cell maturation and function. Eur J Immunol. 2011 Jul;41(7):1883–93.
381. Mazzoni A, Young HA, Spitzer JH, Visintin A, Segal DM. Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. Journal of Clinical Investigation. 2001 Dec 15;108(12):1865–73.
382. Stelekati E, Bahri R, D'Orlando O, Orinska Z, Mittrücker HW, Langenhaun R, et al. Mast Cell-Mediated Antigen Presentation Regulates CD8+ T Cell Effector Functions. Immunity. 2009 Oct;31(4):665–76.
383. Amaral MM, Davio C, Ceballos A, Salamone G, Cañones C, Geffner J, et al. Histamine Improves Antigen Uptake and Cross-Presentation by Dendritic Cells. The Journal of Immunology. 2007 Sep 15;179(6):3425–33.
384. Zaiss DMW, van Loosdregt J, Gorlani A, Bekker CPJ, Gröne A, Sibilia M, et al. Amphiregulin Enhances Regulatory T Cell-Suppressive Function via the Epidermal Growth Factor Receptor. Immunity. 2013 Feb;38(2):275–84.
385. Furukawa H, Kitazawa H, Kaneko I, Kikuchi K, Tohma S, Nose M, et al. Mast Cells Inhibit CD8+ T Cell-Mediated Rejection of a Malignant Fibrous Histiocytoma-Like Tumor: Involvement of Fas-Fas Ligand Axis. Am J Immunol. 2009 Mar 1;5(3):89–97.
386. Nowak EC, de Vries VC, Wasiuk A, Ahonen C, Bennett KA, Le Mercier I, et al. Tryptophan hydroxylase-1 regulates immune tolerance and inflammation. Journal of Experimental Medicine. 2012 Oct 22;209(11):2127–35.
387. Piconese S, Gri G, Tripodo C, Musio S, Gorzanelli A, Frossi B, et al. Mast cells counteract regulatory T-cell suppression through interleukin-6 and OX40/OX40L axis toward Th17-cell differentiation. Blood. 2009 Sep 24;114(13):2639–48.
388. Gri G, Piconese S, Frossi B, Manfroi V, Merluzzi S, Tripodo C, et al. CD4+CD25+ Regulatory T Cells Suppress Mast Cell Degranulation and Allergic Responses through OX40-OX40L Interaction. Immunity. 2008 Nov;29(5):771–81.
389. Gounaris E, Erdman SE, Restaino C, Gurish MF, Friend DS, Gounari F, et al. Mast cells are an essential hematopoietic component for polyp development. Proceedings of the National Academy of Sciences. 2007 Dec 11;104(50):19977–82.
390. Gounaris E, Blatner NR, Dennis K, Magnusson F, Gurish MF, Strom TB, et al. T-Regulatory Cells Shift from a Protective Anti-Inflammatory to a Cancer-Promoting Proinflammatory Phenotype in Polyposis. Cancer Res. 2009 Jul 1;69(13):5490–7.

391. Molfetta R, Paolini R. The Controversial Role of Intestinal Mast Cells in Colon Cancer. Cells. 2023 Jan 31;12(3):459.
392. Varricchi G, Galdiero MR, Loffredo S, Marone G, Iannone R, Marone G, et al. Are Mast Cells MASTers in Cancer? Front Immunol. 2017 Apr 12;8.
393. Yu Y, Blokhuis B, Derks Y, Kumari S, Garssen J, Redegeld F. Human mast cells promote colon cancer growth via bidirectional crosstalk: studies in 2D and 3D coculture models. Oncoimmunology. 2018 Nov 2;7(11).
394. Mao X, Xu J, Wang W, Liang C, Hua J, Liu J, et al. Crosstalk between cancer-associated fibroblasts and immune cells in the tumor microenvironment: new findings and future perspectives. Mol Cancer. 2021 Oct 11;20(1):131.
395. Penninger JM, Irie-Sasaki J, Sasaki T, Oliveira-dos-Santos AJ. CD45: new jobs for an old acquaintance. Nat Immunol. 2001 May;2(5):389–96.
396. Tchilian EZ, Beverley PCL. Altered CD45 expression and disease. Trends Immunol. 2006 Mar;27(3):146–53.
397. Al Barashdi MA, Ali A, McMullin MF, Mills K. Protein tyrosine phosphatase receptor type C (PTPRC or CD45). J Clin Pathol. 2021 Sep;74(9):548–52.
398. Lowell CA. Src-family and Syk Kinases in Activating and Inhibitory Pathways in Innate Immune Cells: Signaling Cross Talk. Cold Spring Harb Perspect Biol. 2011 Mar 1;3(3):a002352–a002352.
399. Zheng X, Li AS, Zheng H, Zhao D, Guan D, Zou H. Different Associations of CD45 Isoforms with STAT3, PKC and ERK Regulate IL-6-Induced Proliferation in Myeloma. PLoS One. 2015 Mar 17;10(3):e0119780.
400. Courville EL, Lawrence MG. Characteristic <scp>CD45RA</scp> / <scp>CD45RO</scp> maturation pattern by flow cytometry associated with the <scp>CD45 C77G</scp> polymorphism. Cytometry B Clin Cytom. 2021 Sep 8;100(5):602–5.
401. Bleesing JJH, Fleisher TA. Human B cells express a CD45 isoform that is similar to murine B220 and is downregulated with acquisition of the memory B-cell marker CD27. Cytometry. 2003 Jan;51B(1):1–8.
402. Oka S, Mori N, Matsuyama S, Takamori Y, Kubo K. Presence of B220 within thymocytes and its expression on the cell surface during apoptosis. Immunology. 2000 Aug;100(4):417–23.
403. Bleesing JJH, Morrow MR, Uzel G, Fleisher TA. Human T Cell Activation Induces the Expression of a Novel CD45 Isoform That Is Analogous to Murine B220 and Is Associated with Altered O-Glycan Synthesis and Onset of Apoptosis. Cell Immunol. 2001 Oct;213(1):72–81.
404. Hermiston ML, Xu Z, Weiss A. CD45: A Critical Regulator of Signaling Thresholds in Immune Cells. Annu Rev Immunol. 2003 Apr;21(1):107–37.
405. Ish-Shalom E, Gargir A, André S, Borovsky Z, Ochanuna Z, Gabius HJ, et al. α2,6-Sialylation promotes binding of placental protein 14 via its Ca2+-dependent lectin activity: insights into differential effects on CD45RO and CD45RA T cells. Glycobiology. 2006 Mar 1;16(3):173–83.
406. Lee CL, Chiu PCN, Pang PC, Chu IK, Lee KF, Koistinen R, et al. Glycosylation Failure Extends to Glycoproteins in Gestational Diabetes Mellitus. Diabetes. 2011 Mar 1;60(3):909–17.

407. Clark MC, Baum LG. T cells modulate glycans on CD43 and CD45 during development and activation, signal regulation, and survival. Ann N Y Acad Sci. 2012 Apr;1253(1):58–67.
408. Earl LA, Baum LG. CD45 Glycosylation controls T-cell life and death. Immunol Cell Biol. 2008 Oct 8;86(7):608–15.
409. Blaylock MG, Lipworth BJ, Dempsey OJ, Duncan CJA, Lee DKC, Lawrie A, et al. Eosinophils from patients with asthma express higher levels of the pan-leucocyte receptor CD45 and the isoform CD45RO. Clinical & Experimental Allergy. 2003 Jul;33(7):936–41.
410. Patterson WP, Caldwell CW, Yesus YW. In Vivo Upregulation of CD45RA in Neutrophils of Acutely Infected Patients. Clin Immunol Immunopathol. 1993 Jul;68(1):35–40.
411. Caldwell CW, Patterson WP, Yesus YW. Translocation of CD45RA in Neutrophils. J Leukoc Biol. 1991 Apr 1;49(4):317–28.
412. Yu CL, Yu HS, Sun KH, Hsieh SC, Tsai CY. Anti-CD45 isoform antibodies enhance phagocytosis and gene expression of IL-8 and TNF-alpha in human neutrophils by differential suppression on protein tyrosine phosphorylation and p56lck tyrosine kinase. Clin Exp Immunol. 2002 Jul;129(1):78–85.
413. Haidl ID, Ng DHW, Rothenberger S, Johnson P, Jefferies WA. Detection of restricted isoform expression and tyrosine phosphatase activity of CD45 in murine dendritic cells. Eur J Immunol. 1995 Dec;25(12):3370–4.
414. Zhang Z, Li J, Zheng W, Zhao G, Zhang H, Wang X, et al. Peripheral Lymphoid Volume Expansion and Maintenance Are Controlled by Gut Microbiota via RALDH+ Dendritic Cells. Immunity. 2016 Feb;44(2):330–42.
415. Clayton K, Vallejo AF, Davies J, Sirvent S, Polak ME. Langerhans Cells—Programmed by the Epidermis. Front Immunol. 2017 Nov 29;8.
416. Wood GS, Szwejbka P, Schwandt A. Human Langerhans Cells Express a Novel Form of the Leukocyte Common Antigen (CD45). Journal of Investigative Dermatology. 1998 Oct;111(4):668–73.
417. Murakami K, Sato S, Nagasawa S, Yamashita T. Regulation of mast cell signaling through high-affinity IgE receptor by CD45 protein tyrosine phosphatase. Int Immunol. 2000 Feb;12(2):169–76.
418. Berger SA, Mak TW, Paige CJ. Leukocyte common antigen (CD45) is required for immunoglobulin E-mediated degranulation of mast cells. J Exp Med. 1994 Aug 1;180(2):471–6.
419. Virts E, Barritt D, Siden E, Raschke WC. Murine mast cells and monocytes express distinctive sets of CD45 isoforms. Mol Immunol. 1997 Nov;34(16–17):1191–7.
420. Rönnberg E, Boey DZH, Ravindran A, Säfholm J, Orre AC, Al-Ameri M, et al. Immunoprofiling Reveals Novel Mast Cell Receptors and the Continuous Nature of Human Lung Mast Cell Heterogeneity. Front Immunol. 2022 Jan 4;12.
421. Kojima M, Nakamura S, Itoh H, Ohno Y, Masawa N, Joshita T, et al. Mast cell sarcoma with tissue eosinophilia arising in the ascending colon. Mod Pathol. 1999 Jul;12(7):739–43.

Annex

Antibodies employed for flow cytometry

Target	Fluorochrome	Clone	Company
CD45	PE-CF594	HI30	BD
CD117	APC	YB5.B8	BD
FceRI	PE-Cy7	AER-37	BioLegend
CD203c	BV421	NP4D6	BioLegend
CD34	BV785	561	BioLegend
Integrin β7	FITC	FIB504	BioLegend
CD45	BV510	HI30	BioLegend
CD14	FITC	M5E2	BD
CD66b	APC	QA17A51	BioLegend
CD16	PE-Cy7	3G8	BioLegend
HLA-DR	PE	1.243	BioLegend
CD11c	BV421	Bu15	BioLegend
CD3	BV510	OKT3	BioLegend
CD4	FITC	RPA-T4	BD
CD8	APC	RPA-T8	BioLegend
CD123	BV785	6H6	BioLegend
CD3	APC	HIT3a	BioLegend
CD19	APC	4G7	BioLegend
CD56	APC	5.1H11	BioLegend
CD14	APC	63D3	BioLegend
CD200	BV421	OX-104	BioLegend
CD44	BV711	IM7	BioLegend