Investigation of Brain CD47 Expression and Its Relation with Cognitive Functions in Diabetic Mice Models

by

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Investigation of Brain CD47 Expression and Its Relation with Cognitive Functions in Diabetic Mice Models

Koç University

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To my father whom I lost before I started my master's degree

ABSTRACT

Investigation of Brain CD47 Expression and Its Relation with Cognitive Functions in Diabetic Mice Models

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Diabetes Mellitus is an endocrine disorder resulting in hyperglycaemia that affects many vital organs of the body like the brain. The detailed molecular basis of dementia and cognitive dysfunction resulting from diabetes is not totally known. CD47 is a cell surface protein working in the homeostasis of the immune system. The relation of CD47 is shown in neurodegenerative diseases. Also, CD47 has a role in pancreatic islet cells and their maintenance in T1DM models. In this thesis, CD47 expression and its relation with cognitive functions is investigated in type 1 and type 2 diabetic mice models induced with STZ and high fat diet respectively. Cognition tests were done to evaluate the animals' cognitive functions. Immunofluorescent staining and imaging were done to investigate the expression of CD47 in hippocampus region of brain tissue. As a result, it is found that there is a significant increase in type 1 and type 2 models in the expression of CD47 levels. There is also a significant difference between two cognitive tests out of three between healthy and T1DM models. Unfortunately, there was no correlation found between CD47 expressions and cognitive functions. To conclude, we think that CD47 levels in the brain may be elevated as a way of cells to protect themselves from excess blood sugar independent from cognitive functions.

Key Words: Diabetes Mellitus, Type 1 Diabetes Mellitus, Type 2 Diabetes Mellitus, CD47, Cognition, Cognitive Functions

ÖZETÇE

Diyabetik Fare Modellerinde Beyindeki CD47 Ekspresyonunun ve CD47'nin Bilişsel Fonksiyonlarla İlişkisinin Araştırılması

Nil Kirişçioğlu

İmmunoloji, Yüksek Lisans

Aralık, 2022

Diyabetes Mellitus yüksek kan şekerinin vücudun beyin gibi önemli organlarını etkilemesiyle sonuçlanan endokrin bir bozukluktur. Diyabetin neden olduğu demansın ve bilişsel bozuklukların moleküler mekanizması tam anlamıyla bilinmemektedir. CD47 immün sistem dengesinde rol oynayan bir hücre yüzeyi proteinidir. CD47'nin nörodejeneratif hastalıklarla ilişki olduğu gösterilmiştir. CD47'nin aynı zamanda tip 1 diyabet modellerinde pankreas adacıklarında da görevi olduğu bazı çalışmalarla gösterilmiştir. Bu tez çalışmasında sırasıyla STZ ve yüksek yağlı diyet ile oluşturulmuş tip 1 ve tip 2 diyabet fare modellerinde CD47 ekspresyonu ve CD47'nin bilişsel bozukluklarla ilişkisi araştırılmıştır. Farelerin bilişsel fonksiyonlarını değerlendirmek için kognitif testler ve beyindeki hipokampus bölgesinde CD47 ekspresyonunu araştırmak için immunfloresan boyamalar yapılmıştır. Sonuç olarak CD47 ekspresyonunun tip1 ve tip 2 diyabet modeli farelerde kayda değer bir derecede arttığı gözlemlenmiştir. Toplamda yapılan üç kognitif testin ikisinde de sağlıklı kontrol fareleri ve tip 1 diyabet modelinde arasında farklılık göze çarpmıştır. CD47 ve bilişsel fonksiyonlar arasında bir korelasyon görülmemiştir. En nihayetinde diyabet modellerinde hücrelerin CD47 seviyesini arttırmayı bilişsel fonksiyonlardan bağımsız bir şekilde yüksek kan şekeri seviyelerinden kendilerini korumak için bir yol olarak seçtiğini düşünmekteyiz.

Anahtar Kelimeler: Diyabet, Tip 1 Diyabet, Tip 2 Diyabet, CD47, Bilişsel Fonksiyonlar

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ABBREVIATIONS

AD	Alzheimer's Disease
ADA	American Diabetes Association
AF	Alexa Fluor

CD47	Cluster of Differentiation 47
CD8	Cluster of Differentiation 8
CRP	C-Reactive Protein
DM	Diabetes Mellitus
GAD65	Glutamic Acid Decarboxylase
GFAP	Glial Fibrillary Acidic Protein
GLUT1	Glucose Transporter 1
GLUT2	Glucose Transporter 2
H&E	Haematoxylin & Eosin
HbA1c	Haemoglobin A1c
HF	High Fat
HLA	Human Leukocyte Antigen
IA-2A	Protein Tyrosine Phosphatase
IAA	Insulin Autoantibody
ICA	Islet Cell Autoantibody
IDF	International Diabetes Federation
IF	Immunofluorescent
IFN	Interferon Gamma
IHC	Immunohistochemistry
IL-6	Interleukin-6
IP	Intraperitoneal
KUARF	Koç University Animal Research Facility
LASX	Leica Application Suite X
MS	Multiple Sclerosis
NGF	Nerve Growth Factor
NOR	Novel Object Recognition
OF	Open Field
OGTT	Oral Glucose Tolerance Test
PD	Parkinson's Disease
RT	Room Temperature
SIRP	Signal Regulatory Protein Alpha
STZ	Streptozotocin

T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TSP-1	Thrombospondin-1

Chapter 1:
INTRODUCTION

1.1 Diabetes

Diabetes Mellitus (DM) is an endocrine and metabolic disorder in which glucose entry into the cells are defected and that is believed to have 643 million cases by 2030 [IDF, 2021]. Glucose is the key element for energy production of the body. All big molecules are broken down into glucose to be processed by the cell for ATP production. When the glucose levels are high in the blood, insulin is secreted to signal cells to take up glucose.

Hence, insulin hormone, together with glucagon hormone, regulates blood glucose levels. When insulin secretion or this signalling of cells are impaired, DM occurs [Nakrani, 2022].

Insulin is secreted by the β-cells found in the islets of pancreas. Preproinsulin, precursor of the insulin, is secreted as the β-cells sense the change in plasma glucose via glucose transport 2 (GLUT2). Preproinsulin is then processed into proinsulin and lastly to insulin and stored [Fu, 2013]. Stored insulin is sent to the bloodstream to trigger cells to take up glucose. Secretion of insulin and this signalling of cells may fail to function properly in different ways leading to various types of DM.

There are two main more common types of DM which are type 1 and type 2 and there are other rare types such as gestational DM and other disease related DM. According to American Diabetes Association (ADA) T1DM is the T-cell mediated autoimmune type of DM in which auto-antibodies destruct the β-cells and causing insulin deficiency whereas the definition of T2DM is the progressive loss of β-cell activity together with an insulin resistance [ADA, 2021]. Gestational DM is diagnosed during pregnancy, second or third trimester, in people having no symptoms or disease history prior to pregnancy. Disease related DM comprise monogenic diabetes syndrome, exocrine pancreas diseases and drug usage induced DM.

Having less T1DM cases than T2DM, the prevalence of the disease has increased in the last years. International Diabetes Federation (IDF) 2021 data suggests 541 million people are in risk of diabetes [IDF, 2021]. The rate of increase of the cases is 90% in 12 years according to research [Satman, 2013]. It is believed that the DM cases will rise to 783 million by the year 2045. In the year 2021, the expenditure for DM is assumed to be 966 billion dollars [IDF,2021]. Hence this disease is one of the costliest diseases and has economic burden both for the patient and the country. Not only the treatments are expensive for the patient, it is hard for government to compromise and support the treatments of the patients. That is why further research on DM should be taken into serious consideration.

1.1.1 Pathophysiology

Type 1 Diabetes Mellitus

T1DM is seem to generally develop in young ages; although cases with T1DM development in old ages exists. The disease has a genetic background and the contribution of environmental factors. The current research suggest that HLA genes are the main inheritance way. Class II HLA loci is the major risk factor together with autoantibodies increasing the susceptibility of the disease.

One of the hallmarks of T1DM is insulitis. Insulitis is the destruction and apoptosis of the ß-cells by the infiltration of immune cells into the islets. $CD8^+$ T cells which can recognize GAD65 and IA-2A are the mostly found cells in the area but B cells, $CD4^+$ T cells and macrophages also accompany $CD8^+$ T cells for destruction. [Ilonen, 2019].

Type 2 Diabetes Mellitus

Environmental factors and genetic factors are the risk factors for T2DM. An unhealthy diet and an absence of physical activity affects the glucose metabolism. It is found that physical activity can enhance glucose uptake via increasing blood flow and can reduce the abdominal fat to increase insulin sensitivity [Galicia-Garcia,2020].

Obesity which is considered as having a body-mass index of equal or higher than $30kg/m^2$, is known to be one of the main contributors to diabetes. Although the exact relationship and the mechanism underlying still remain to be found, obesity is seemed to be associated insulin resistance. Normally after glucose levels increase in the blood after a meal, insulin is secreted and blood levels of insulin increases. Uptake of glucose of adipose and muscle cell is initiated by along with increasing insulin and gluconeogenesis inside the cells starts. Insulin resistance is known as the cell's loss of its sensitivity to insulin circulating in the blood. Chronic low level inflammation and a resulting oxidative stress is initiated in the light of obesity. Elevation of some factors such as TNF-α, IL-6 and IFN-γ are seen in this inflammation in adipose tissue of obese patients. This elevation impairs the insulin sensitivity and causes a metabolic dysfunction. Immune cell infiltration, mostly macrophages, is seen in obese patients as these factors increase. However, removal of some of these factors are found to ameliorate this inflammation and insulin resistance [Jeong-Ho, 2009].

1.1.2 Diagnosis of Diabetes Mellitus

Diabetes starts with hyperglycaemia which can be detected via measuring blood glucose levels. Fasting plasma glucose is the blood sugar level after 8 hours of fasting and it is first measure to be checked in the diagnosis of DM. A 100-125 mg/dL fasting blood glucose level is an indication of insulin resistance whereas a fasting blood glucose level of 126 mg/dL or higher is considered as DM. If the fasting plasma glucose is not significantly higher than, oral glucose test (OGTT) is needed to be done to potential patients. In OGTT, blood sugar levels are again measured after giving a glucose bolus to the patient. In this test, a result of 140-199 mg/dL is a measure of metabolic syndrome and 200 mg/dL or higher is an indication of DM.

Haemoglobin A1c levels (HbA1c) are also important for the diagnosis of DM. HbA1c is the glycosylated form of haemoglobin circulating in the blood. A 5.7%-6.4% HbA1c is considered as prediabetes and 6.5% or higher is considered as DM (Table 1).

In order to distinguish T1DM from T2DM, the circulation of special autoantibodies in the bloodstream can be checked. Four autoantibodies are found to be related with T1DM and are used in the diagnosis of the disease: islet cell antibodies, ICA; glutamic acid decarboxylase, GAD-65; insulin autoantibodies, IAA and protein tyrosine phosphatase, IA-2A. In T1DM patients either one or some of the antibodies are positive [Pippitt, 2016].

Table 1-1: ADA Criteria for the Diagnosis of DM

Test	Level	Condition
Fasting Blood Glucose	100-125 mg/dL	Prediabetes
	≥126 mg/dL	Diabetes
OGTT	140-199 mg/dL	Prediabetes
	≥ 200 mg/dL	Diabetes
HbA1c	5.7-6.4%	Prediabetes
	>6.5%	Diabetes

Abbreviations: ADA: American Diabetes Association; OGTT: Oral Glucose Tolerance Test; HbA1c: Haemoglobin A1c

Currently, there are also existing studies that are trying explore new diagnostics in the onset of DM. Elevation of C-reactive protein (CRP) had grabbed attention in some studies. High-sensitive CRP levels seem to elevate and there is a positive correlation with high sensitive CRP levels and HbA1c levels [Tütüncü, 2016].

1.1.3 Current Treatments

The current treatment is the usage of exogenous insulin for T1DM, either insulin injections or insulin pump, together with proper diet, carbohydrate counting, frequent blood sugar measurements and exercise. Similar to T1DM, a treatment for T2DM also includes healthy diet with the intake of food with low glycaemic index and physical activities. Instead of insulin usage, oral diabetes drugs such as metformin are used. Prediabetes and the onset of DM can be reversed by increasing daily physical activity and having a healthier diet reducing salt and sugar intake [Skyler, 2017].

1.1.4 Effects of Diabetes Mellitus

DM is found to affect not only blood sugar levels but also other vital parts of the body such as brain and heart. There is evidence that people with DM has higher chance to develop cognitive problems and trigger Alzheimer's disease. It is suggested that DM may lead to excessive phosphorylation of Tau protein that is the key protein in the progression of AD. In addition, the excess glucose circulating in blood and insulin resistance may damage brain leading to dementia and memory loss [Jellinger, 2015]. On the other hand, high glucose is needed during times with high energy demand such as learning and unfortunately the utilization of it decreases with age. Although it is believed that the brain uptake of glucose is not mainly dependent on insulin, insulin keeps the uptake under control which is important for future glucose usage. Astrocytes are also pointed in brain insulin resistance. They have insulin receptors that keeps the GLUT1 receptors on the plasma membrane to regulate glucose utilization. It is suggested that the alteration of these regulations and glucose metabolism results in memory impairment. However, there is still so many question marks about the interaction of T2DM aging and leading to cognitive dysregulation and dysfunction [Garcia Serrano, 2020].

In addition to cognitive problems, DM can also lead to cardiovascular problems. The highest morbidity in DM is caused by cardiovascular problems such as myocardial infarction, coronary artery disease or atherosclerosis. People with DM is shown to have higher blood pressure than healthy people. It is also suggested that people with DM has higher chance of having cardiovascular problems. Although the exact mechanism is unknown, epigenetic factors may have a role along with oxidative stress, intercellular and metabolic changes caused by hyperglycaemia [Matheus, 2013].

Retinopathy is another most frequent effect of DM. Almost all T1DM and T2DM patients have retinopathy and loss of vision. Increase in blood sugar induces the risk of retinopathy. It is showed that ameliorated and controlled blood sugar decreases the risk retinopathy [Donald, 2004].

1.1.5 Animal Models of Diabetes Mellitus

There are several experimental mice and rat models to study DM. It can be induced either with genetic manipulation or with drugs, chemicals and special diets. For induction of T1DM Alloxan or Streptozotocin can be used. STZ at low doses triggers an immune reaction. It is recognized by the GLUT2 receptors found on β-cells since it consists of a glucose moiety [Lenzen, 2008]. It destructs cells, triggers their necrosis and immune cell infiltration for their phagocytosis [Graham, 2011]. Special mice species and rat species have mutations that mimics T1DM. Genetically induced and engineered animals such as db/db mice and Zucker fatty rats are also used for T2DM and T1DM. In order to induce T2DM high fat diets can also be used [Kottaisamy, 2021]. Although it requires a longer period of time to develop diabetes, usage of high fat diet has the advantage of mimicking obesity like humans.

1.2 CD47

Cluster of differentiation 47 (CD47) is a transmembrane glycoprotein. It functions in the regulation of the immune system homeostasis and macrophage mediated phagocytosis. On healthy cells, it works as a “Don’t eat me signal!” to prevent the phagocytosis of the

cell by macrophages (Figure 1.1). It is found to be mainly expressed on red blood cells and other cell types with difference in the expression levels [Hayat, 2020].

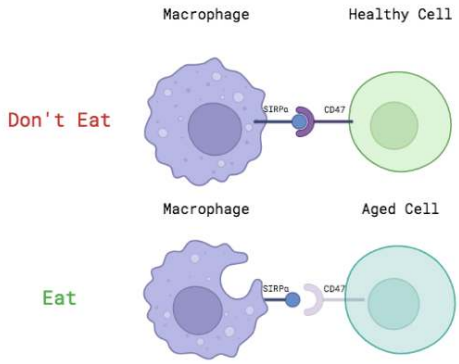


Figure 1.1: Function of CD47 as "Don't Eat Me!" signal

The figure was created by BioRender.

In aged cells CD47 expression is downregulated whereas in healthy cells it is upregulated. The expression level of CD47 not only vary with the age of the cell but also with tissue and some pathological conditions. Depending on The Human Protein Atlas data; brain tissue, specifically cerebral cortex and cerebellum areas, has the highest CD47 expression whereas colon tissue has the lowest. Some tissues also have no detection of CD47 expression.

1.2.1 CD47 Interactions

CD47 has several ligands such as integrins, thrombospondin-1 (TSP-1) and signal regulatory protein alpha (SIRPα). The cell type and the ligand decides the faith of the transduction and the downstream events. TSP-1 interaction with CD47 results in the regulation of processes like angiogenesis. SIRPα is also transmembrane glycoprotein like CD47 and is expressed on neuronal cell and myeloid antigen presenting cells such as monocytes, macrophages, myeloid dendritic cells and granulocytes. SIRPα and CD47 interaction regulates the immune system homeostasis. The interaction keeps healthy cells in blood circulation by preventing their phagocytosis rather than their clearance from the body whereas the absence of interaction results in the macrophage mediated phagocytosis of old cells [Hayat, 2020].

1.2.2 CD47 Expression in Pathological Conditions

CD47 expression is found to differ in some pathological conditions such as cancer and neurodegenerative diseases. In some cancer types such as multiple myeloma, acute lymphoblastic leukaemia and mouse myeloid leukaemia, the expression of CD47 is reported as increased as a principal mechanism of cancer cells to escape from immune cells from being phagocytosed [Jaiswal, 2009].

CD47 is also one of the key modulators in the case of neurodegenerative disorders like MS, AD, stroke and brain injuries. It is found to function in both recovery of injuries and development of the diseases and in neuron death. CD47 uses phagocytosis as the primary mechanism in an autoimmune neuroinflammation. In MS cases, there is a downregulation of CD47 in mRNA and protein levels. Junker et al. suggested that dysregulation of microRNAs reduces CD47 levels leading to promotion of phagocytosis of cells [Junker, 2009]. Research done on CD47 knockout mice revealed that a decrease in damage of ischemic brain injury and reduction in neutrophil extravasation to brain. For stroke treatment C47 may be an anti-inflammatory target. Blocking of CD47 seems to reduce the damage after a brain haemorrhage and speeds up hematoma clearance [Gheibihayat, 2021].

Table 1-2: Neurodegenerative Diseases and their relation with CD47

Neurodegenerative Disease	**CD47 Effect**
Stroke	Stimulates the disease
MS	Initiation and progression
AD	Stimulates the disease
PD	Protection
Spinal Cord and Brain Injuries	Stimulates the disease, disease progression and promotion of immune cell infiltration

Abbreviations: MS: Multiple Sclerosis; AD: Alzheimer's Disease; PD: Parkinson's Disease

Besides cancer and neurodegenerative diseases, an effect and a change of CD47 is also found to be in pancreatic tissue in diabetes cases. There is evidence showing that CD47

is having a role in the inflammation of pancreatic islet cells in the case of T1DM. It is suggested that in STZ-induced T1DM mice models, CD47 levels decrease, resulting in reduced CD47/SIRP alpha interaction and leading to infiltration of macrophages into the islets and finally the destruction of the tissue and insulin secretion [Zhang, 2020].

1.3 Cognition and Cognitive Functions

Cognitive functioning refers to multiple mental abilities; including learning, thinking, reasoning, remembering, problem solving, decision making, and attention. The brain is the main organ responsible for the cognitive functions. Being the largest part of the brain, cerebrum is the centre for initiation of movement, speech, learning, reasoning and thinking. Cerebrum consists of four lobes divided as frontal, parietal, temporal and occipital lobe each having important roles in cognition. The butterfly shaped part, viewing in coronal sections, under temporal lobe is known as the hippocampus and is known to be related with memory and learning. Hippocampus is shown to express high levels of insulin receptors. The cognition performance of it as in memory and learning depends on adequate amount of glucose [Ewan, 2010]. It is found that diabetic conditions lead to synaptic impairments and defective neurotransmission and therefore hippocampal plasticity. This change in the integrity of the hippocampus is accompanied by an increase in the number of astrocytes, known as astrogliosis, and neuroinflammation [Garcia Serrano, 2020].

The gradual loss of function and change of neurons is known as neurodegeneration. Dementia is the result of malfunctioning of the nerve cells in the brain leading to a decline in the cognitive functions. Alzheimer's Disease is the most common progressive dementia type.

1.3.1 Nerve Growth Factor & Glial Fibrillary Acidic Protein

Nerve Growth Factor (NGF) is a neurotropic factor that has a role in the maintenance and survival of neurons. Cholinergic neurons are the cells that degenerate in AD. It is believed that neurotrophic factors have an important role in neurodegenerative disorders such as

AD and dementia since they support the growth of neurons. Pharmacologically usage of NGF may ameliorate the cholinergic neurons and may become a possible treatment [Jönhagen, 2000].

Glial Fibrillary Acidic Protein (GFAP) is uniquely found in astrocytes and is an intermediate filament type III protein. It has a major role in supporting the cytoskeleton of astrocytes and activating them. So, it is used as a marker of astrocytes. A mutation in GFAP is associated with a disease called Alexander Disease which grabs the relationship of GFAP to other neurodegenerative disorders. Active astrocytes are found to surround amyloid plaques which are one of the main causes of AD [Yang, 2015].

1.3.2 Cognition Tests used in Animal Experiments

Rodents are being used for research in neurodegenerative diseases and cognitive dysfunction. There are several tests developed to study and assess cognitive abilities of mice and rats. Y-Maze, Social Discrimination, Novel Object Recognition, Olfactory Discrimination and Open Field tests are the most known and used cognitive tests [Hölter, 2015]. In this research Open Field, Y-Maze and Novel Object Recognition tests were used.

Open Field is test for the evaluation of emotions, behaviours and responses to outer stimuli. In this test, a box 50cmx50cmx38cm box with grid floor is used. The animal is put in the box for 5 minutes and its behaviours are recorded. There are many parameters checked in this test depending on the experiment. The distance travelled, number of squares passed, number of faeces done, the time spent in inner squares or outer squares of the box can be evaluated and give information about the cognitive functions of the animal [Seibenhener, 2015].

Spontaneous Alternation in the Y-Maze is a test depending on the environmental experimentation of the rodents. In this test, a Y-shaped box with three identical arms placed 120° next to each other is used (Figure2.3). The animal is put in the box for 5 minutes and its movements and the arms visited are recorded. Healthy mice tend to explore the least visited arm remembering the recently visited arm having a successful combination of three arm visits. On the other hand, a mouse with cognitive impairment

forgets the arm it visits and have less combination of three arms named as spontaneous alternation. This test evaluates the memory and attention [Hölter, 2015].

Novel Object Recognition test is done to evaluate the animals' tendency to recognize an unfamiliar object and for assessing visual memory. It consists of two steps which are the pretest and test steps. In the pretest step the animal is put in a box with two identical objects and allowed to explore both objects for 10 minutes. After 6 hours, test step is done. In the test step, one of the objects is changed with a novel one (Figure 2.4). The novel object is important in terms of its shape and look. The animal should not be biased and should not explore the object just because its attention-grabbing look rather than it being novel [Hölter, 2015]. The test step is 5 minutes and the time animal spent on both objects are recorded. The healthy animal should be able to recognize the former object therefore; it is expected to spent more time on the novel one. Contrary to healthy, cognitive impairment should result in similar time spent on both former and novel objects [Leger, 2013].

1.4 Aim

The molecular mechanism under dementia, memory loss and dysfunction of cognition caused by DM is still not very clear. Therefore, the aim of this thesis research is to examine the CD47 expression in brain tissue of healthy and diabetic mice to check whether there is a difference that may give hints about the underlying molecular mechanisms. Since CD47 is found to have a function both in neurodegenerative diseases and T1DM pathogenesis, we thought that it may also has a role in DM resulted dementia and memory loss. Hence the memory region of the brain, the hippocampus, was checked with IF staining. The cognitive functions of the mice were also evaluated with three types of tests to see if there is a correlation between cognitive functions and CD47 expressions in STZ induced T1DM and HF diet induced T2DM models.

Chapter 2:
MATERIALS & METHODS

2.1 Animal Models

The certificate for the usage of animals in research were given by Istanbul University after completing 80 hours of theoretical and practical education.

The approval for animal research was given from Local Ethical Committee (Approval number: 2021-30).

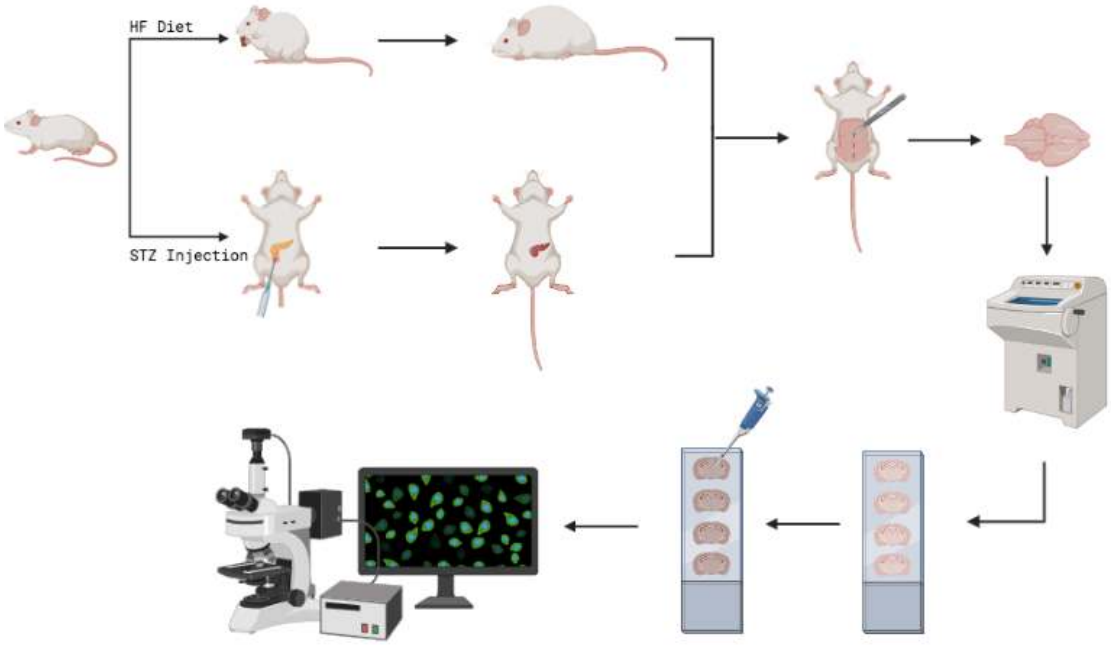


Figure 2.1: Overview of the Experimental Setup

The figure was created by BioRender. Abbreviations: HF: High Fat; STZ: Streptozotocin

2.1.1 Construction of Diabetic Mice Models

CD1 male, 7-week old mice which were born on 14.04.2022 in Koç University Animal Research Facility, KUARF, were used as T1DM models. The animals were maintained in Tecniplast GM500 IVC cages with a 12hr:12hr light/dark cycle. 36 mice were divided into three groups as Control, and T1DM. T1DM mice were injected with STZ intraperitoneally with a dosage of 50mg/kg for five consecutive days for T1DM induction. After each injection, subcutaneous isotonic solution was given to mice. Since STZ causes a rapid destruction, the mice can suddenly die because of hypoglycaemia. Therefore, in order to minimize this, after each injection mice were also provided water with 10% sucrose [Wu, 2008]. T1DM and control mice were fed Kliba Nafag 3430 Maintenance Standard ad libitum.

CD1 male, 7-week old mice which were born on 15.02.2022 in Koç University Animal Research Facility, KUARF, were used as T2DM models. The animals were maintained in Tecniplast GM500 IVC cages with a 12hr:12hr light/dark cycle. 24 mice were divided into two groups as Control and T2DM. T2DM group mice were fed with Kliba Nafag 2127 Experimental, purified High Fat Diet: 60 % kcal % fat ad libitum. Every week the weight of the animals was measured with Dikomsan Scale and recorded.

2.1.2 Blood Sugar Measurement and Oral Glucose Tolerance Test

Blood sugar measurement was done every week to determine the metabolic syndrome/diabetes conditions of T1DM, T2DM and control mice. The blood sugar measurement was done from tail tip blood with Roche ACCU-CHEK Instant and its competent strips.

Oral glucose tolerance test was done at week 11 for diabetes diagnosis. 2gr/kg glucose bolus were given to mice by oral gavage with 12# 55mm gavage needle and the blood sugar data were recorded at 0, 15, 30, 60, 90, 120 minutes.

2.1.3 Cognition Tests

Open field, Y-maze and Novel Object Recognition cognition tests were done on mice one day before sacrificing the mice.

Open field (OF) test was done using a square empty black box with a white grid field (Figure 2.2). Mice were put in the box for 5 minutes and their movements were recorded. Number of the squares which the mice passed were counted.

Figure 2.2: Open Field Cognition Test Setup

Y-maze test was done using an empty Y shaped box; three arms separated 120° apart each other (Figure 2.3). Mice were put in the box for 5 minutes and their movements were recorded. The order of the arms the mice entered was recorded. Each arm is named as 1,2 and 3. The order of the arms visited were noted along with the number of arms visited and number of successful combination of three arms to calculate spontaneous alternation percentage.

Figure 2.3: Y-Maze Cognition Test Setup

Novel Object Recognition (NOR) is a two-step cognition test. In the first step the mice were put in a square black box with a white grid field and subjected to two exact objects for 10 minutes. After 6 hours, the mice were put in the same box with one of the former object and a novel object (Figure 2.4). The time spent with both of the objects of the mice were recorded.

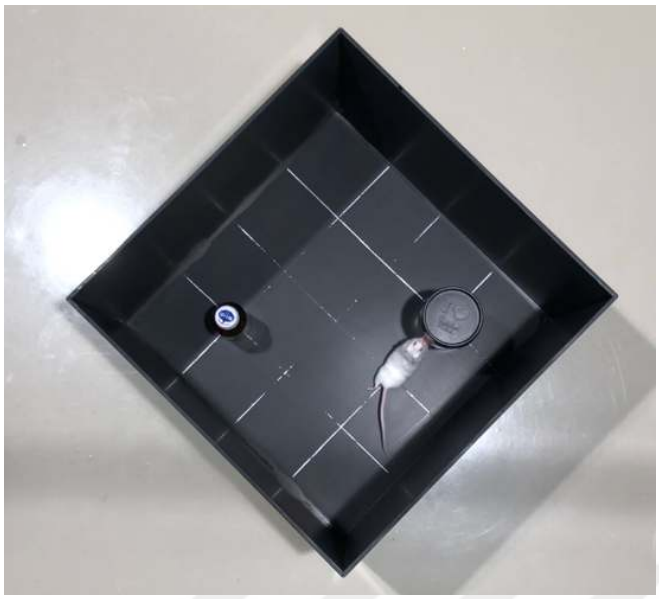


Figure 2.4: Novel Object Recognition Test Setup

2.1.4 Sacrificing Mice and Collection of the Organs

As anaesthetics, mice inhaled 5% induction dosage of isoflurane and after induction, the dosage was lowered to 1-3% for maintenance. Following anaesthesia, mice were sacrificed by exsanguination and the organs were taken and weighed and were collected in 4% PFA for tissue fixation. After one day of PFA fixation the brains were transferred to firstly 10% sucrose solution, then 20% sucrose solution and lastly 30% sucrose solution, fixed in each for one day. Then the brains were cut into three parts and were buried in OCT to store at -80°C until cryosectioning. Pancreas of the mice were also fixed in 4% PFA for one day and buried in paraffin.

2.2 Stainings

2.2.1 Cryosectioning

10µm cryosections of mice brain were taken with Leica CM1950 Cryostat for immunofluorescent staining of the tissue. The sections were put on Epredia Superfrost™ Plus Adhesion Microscope Slides and stored in -80°C until staining.

2.2.2 Immunofluorescent Staining

For an effective IF staining, antigen retrieval with Abcam Antigen Retrieval Buffer (Tris-EDTA Buffer, pH 9.0) was done. The tissues were subjected to heat in the buffer using microwave oven for 2 minutes and cooled for 10 minutes. After cooling down, they were dipped in absolute methanol for five minutes. The tissues were then washed with DPBS and surrounded with hydrophobic barrier pen, Pap-Pen. ScyTek Laboratories Inc. Superblock was put on the tissues and waited in RT for an hour for blocking. Following that, sections were incubated with Abcam Primary Rabbit Polyclonal anti-CD47 antibodies, ab175488, that were diluted 1/200 in the same Superblock at 4°C overnight. The cryosections then were washed three times with DPBS and incubated with Alexa Flora goat anti-rabbit 514 secondary antibodies that were diluted 1/200 with the same Superblock for 1 hour at 37°C. The sections were again washed three times with DPBS. Washed sections were then covered with Abcam ab104139 Mounting medium with DAPI and cover slides lastly.

2.2.3 Microscopic Imaging

After immunofluorescent staining the sections and the stainings were visualised with Leica DMI8-SP8 Confocal Microscope and Leica Application Suite (LASX) software. 405, 488 and 514 solid lasers and PMT1 and PMT2 detectors were used. The images were taken under 20X magnification, 0.75 zoom, 2048x2048 format and Frame Accumulation 2 settings. Z-stack videos were taken with 20 number of steps and maximum projection images with reduced background noise were used for further image analysis.

2.2.4 Immunohistochemistry

Thermo Fisher Scientific UltraVision Detection System Anti-Polyvalent, HRP/AEC TP-015-HA kit was used for IHC staining. To deparaffinise the sections, they were put in toluene for 30 minutes and then to decreasing concentrations of ethanol (100% and 70%), 2 minutes in each, and lastly to distilled water. The sections were washed with PBS for 10 minutes. After washing, they were treated with 3% hydrogen peroxidase for endogenous peroxidase activity blocking for 10 minutes and then washed with PBS for three times. The sections were subjected to Ultra V Block for 5 minutes to minimize non-specific binding. To either stain the pancreas tissues with insulin or glucagon, the tissues

were incubated with mice monoclonal insulin antibodies, diluted 1:5000, overnight at 4°C. Next day, they are washed again three times with PBS, incubated with biotin tagged secondary antibody for 10 minutes and again washed three times. Streptavidin was then applied on the sections for 10 minutes and washed three times. The sections treated with Substrate-chromogen for 5 minutes and washed with distilled water this time. To counterstain hematoxylin was used for 45 seconds and it was washed under running tap water. Lastly the sections were washed with PBS and distilled water and covered with glycerine gel and visualized using Leica DM 2500 light microscope and LASX software.

2.2.5 Haematoxylin & Eosin Staining

The sections were first washed with distilled water for H&E staining. Then they were stained with Haematoxylin for 15 minutes, were washed with tap water and then with 70% ethanol-HCl solution. The sections were again washed with tap water for 15 minutes. After washing they were stained with eosin for 3 minutes and were again washed with tap water. The sections were dehydrated with increasing ethanol concentrations (70%, 95% and 100%), 2 minutes in each and with toluene. Lastly, they were covered with Entellan.

2.3 Statistical Analysis

2.3.1 ImageJ

CD47 stained cells were counted and Java ImageJ image processing programme was used for the analysis of the stained areas. The images were splitted into channels based on the colour of the stainings and both the area of green and blue stainings and the overlapped stainings are measured.

2.3.2 SPSS & GraphPad Analysis

GraphPad Prism 8 software was used for the statistical analysis of weight, blood sugar, OGTT, cognition test and imageJ data. Mann-Whitney and Kruskal Wallis tests for used to show statistical significance of the data. The data are presented as Mean±SEM. $p<0.005$ are considered as significant.

For correlation analysis IBM SPSS Statistics Version 26 was used. Spearman's rho was used for measuring the association between CD47 expressions and cognitive functions.

Chapter 3:
RESULTS

This research was done using 39 CD1 male mice which are grouped as control (n=12), T1DM (n=11) and T2DM (n=16).

3.1 Animals

The weights of the mice were recorded every week. Control group showed a stable trend. T1DM group mice seemed to lost weight in the beginning and then gained weight hence; there was no statistical difference between the weight of control and T1DM mice (p=0.127). T2DM group showed an increasing trend starting from the third week. Comparing three groups, specifically after the fourth week, the difference of the weights between T2DM and control becomes significantly noticeable (p=0.0061) (Figure 3.1).

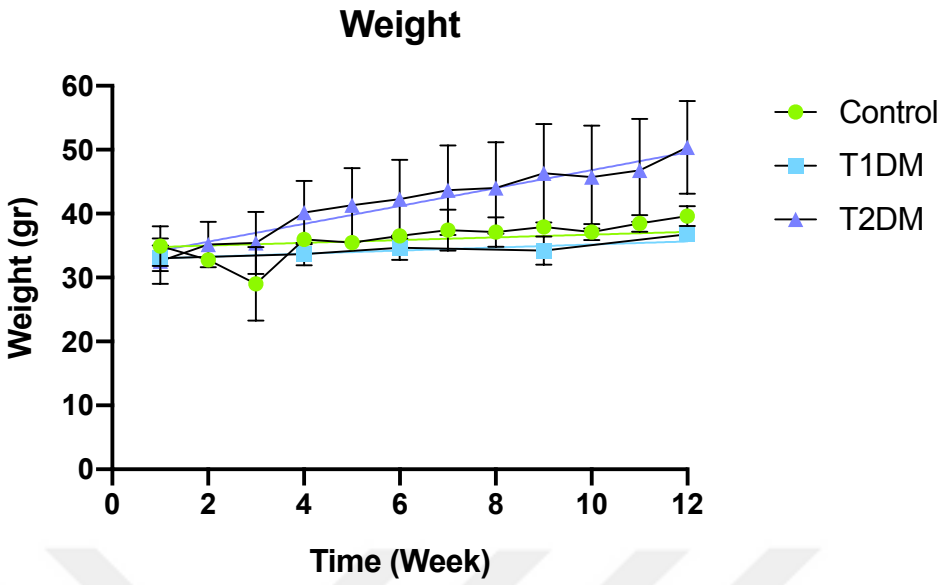


Figure 3.1: Weight of the mice recorded every week in grams

Data are presented in Mean ± SEM. The weight of the mice started to differ after fourth week. There is a significant difference between the weight of control and T2DM groups.
Abbreviations: T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

Also, exposure to HF diet led the mice to obesity. After sacrificing the mice, it was seen that T2DM mice had high amount body fat compared to control mice, which is the main reason for increased body weight (Figure 3.2). Adipose tissue was present almost everywhere in the body including vicinity of the heart, lungs (Figure 3.3a) and intestines (Figure 3.3b).

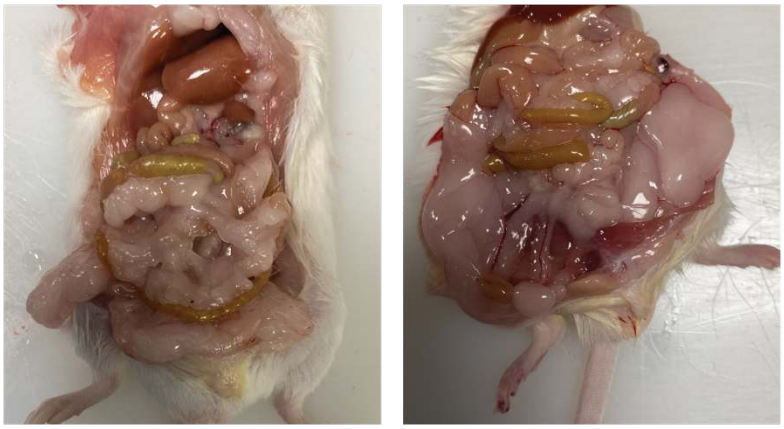
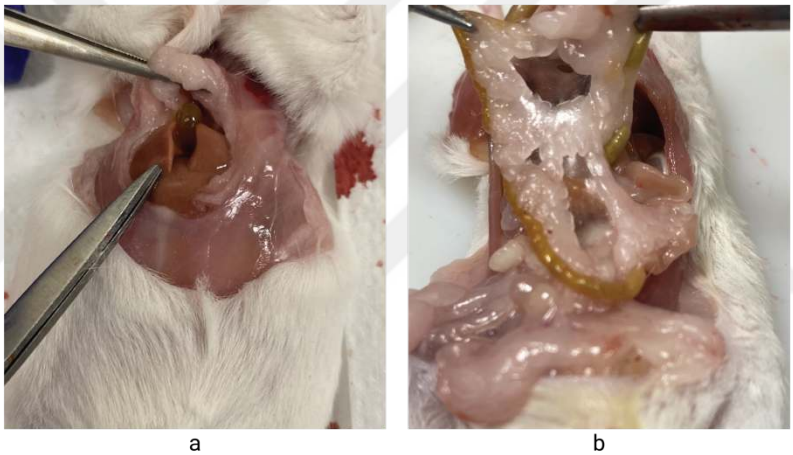


Figure 3.2: Body fat of T2DM mice



a b

Figure 3.3: (a) Body fat surrounding heart and lungs (b) Body fat surrounding intestines

Blood sugar of the mice was measured from the tail tip blood every week. 180-200mg/dL 6 hour fasting blood sugar was considered as metabolic syndrome. 200mg/dL and higher 6 hour fasting blood sugar was considered and diagnosed as Diabetes Mellitus. Just before sacrificing mice, control group had minimum blood sugar of 90 mg/dL and maximum of 157mg/dL. On the other hand, T2DM group had minimum of 144 mg/dL and maximum of 214mg/dL whereas T1DM mice had minimum blood sugar of 205 mg/dL and maximum blood sugar of 501 mg/dL. Starting from week 2, the blood sugar of T1DM group increased significantly ($p= 0.004$) following STZ injections. The control and T2DM groups had similar blood sugar levels, T2DM mice with higher blood sugar levels (Figure 3.4). Chronic exposure to HF diet developed hyperglycaemia in T2DM.

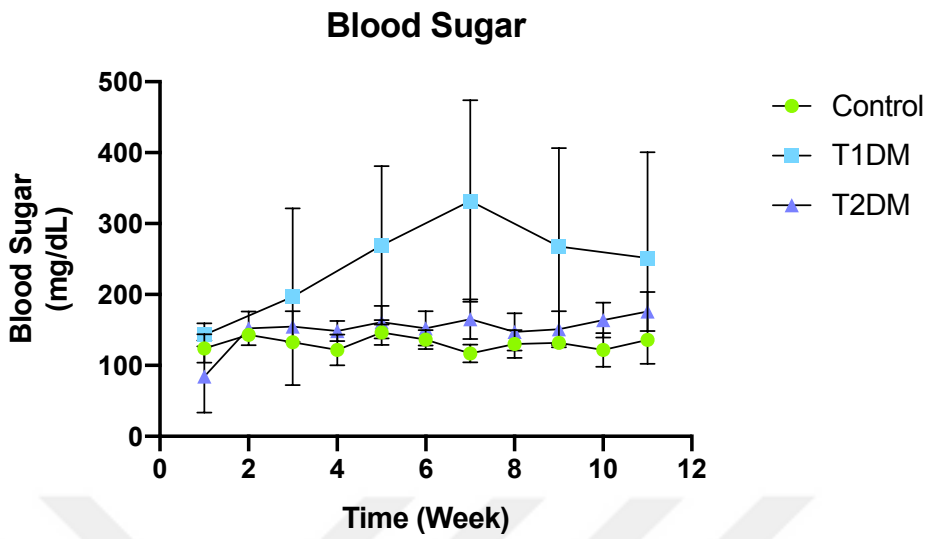


Figure 3.4: Blood sugar levels of three groups measured weekly in milligrams over deciliters

The data are presented in Mean ± SEM. The blood sugar levels have showed a difference starting from week 2. There is a significant difference between T1DM and other groups.
Abbreviations: T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

Oral glucose tolerance (OGTT) test was done at the end of week 11 to diagnose the T2DM mice with Diabetes Mellitus and to show control mice are healthy. Since T1DM mice have already had very high blood sugar levels, OGTT was not done them. After giving the glucose bolus, the blood sugar levels of mice increased immediately. At t=15min, the blood sugar levels of control mice were around 200 mg/dL whereas blood sugar of T2DM mice were almost around 300mg/dL. After t=30min, the blood sugar levels of both groups showed a decreasing trend however, T2DM mice blood sugar glucose levels remain higher around 350mg/dL than control group levels which was around 150mg/dL. Considering the OGTT results, the control group mice can be proven as healthy. On the other hand, T2DM can be diagnosed with Diabetes Mellitus (Figure 3.5).

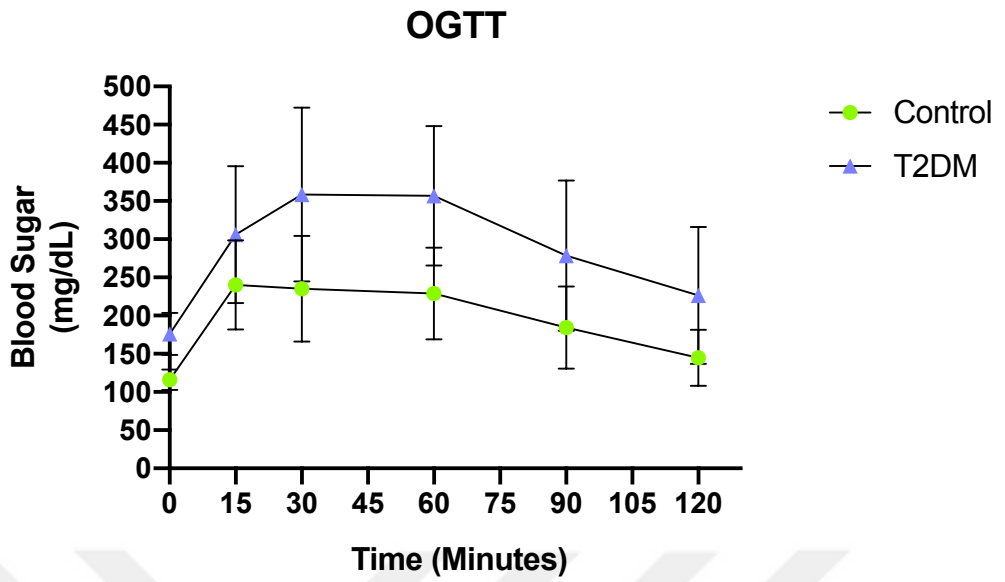


Figure 3.5: Oral Glucose Tolerance Test Results

The data are presented in Mean ± SEM. The blood sugar levels started to increase after 15 minutes of glucose bolus injections. There is a significant difference between T2DM and control group.
Abbreviations: OGTT: Oral Glucose Tolerance Test; T2DM: Type 2 Diabetes Mellitus

3.2 Cognition Tests

Three types of cognition tests were performed on control and T1DM groups one day before sacrificing the mice to see cognitive functions of the mice and how they are affected with DM. Unfortunately, T2DM group mice started to die unexpectedly after OGTT. Hence, no cognition tests were performed on mice with T2DM.

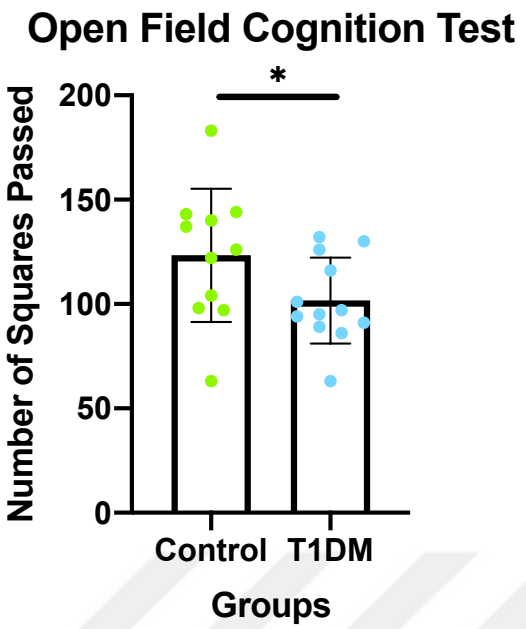


Figure 3.6: Open Field Cognition Test Data

The graph shows the number of squares passed by each mouse in control and T1DM groups. The number of squares control group mice has passed is significantly higher than the T1DM group. Abbreviations: T1DM: Type 1 Diabetes Mellitus

For Open Field test, number of the squares the mice passed was calculated. The number of squares passed was higher in control group ($\bar{x}$= 123.4) compared to T1DM ($\bar{x}$= 101.6). There was a significant difference between the groups (p= 0.03) (Figure 3.6).

For Y-Maze test, the order of the arms mice visited was recorded. Each three visit was considered as a round. Possible rounds were calculated based on the number of arm visits. Alternation rounds were calculated based on the order of the visits. Percentage of alternation was then calculated by dividing alternation rounds to possible rounds and multiplying with 100. There was a difference between control group ($\bar{x}$= 52.2) and the T1DM group ($\bar{x}$= 49.05) but the difference was not significant (p=0.61) (Figure 3.7).

For Novel Object Recognition, the time spent on both former and the novel objects was recorded to calculate discrimination index. There was a significant difference in discrimination index between control ($\bar{x}$= 0.56) and T1DM groups ($\bar{x}$= 0.38) (p=0.01) (Figure 3.8).

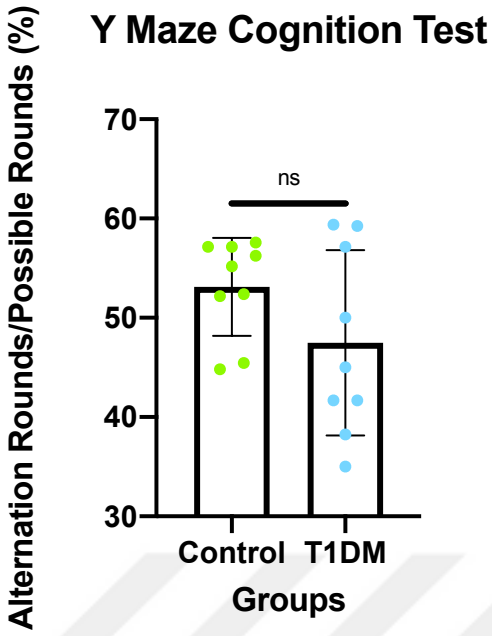


Figure 3.7: Y-Maze Cognition Test Results.

The graph shows the percentage of alternation/possible rounds of each animal in control and T1DM groups. There was a variety of results in T1DM group so there was no significance between the groups. Abbreviations: T1DM: Type 1 Diabetes Mellitus

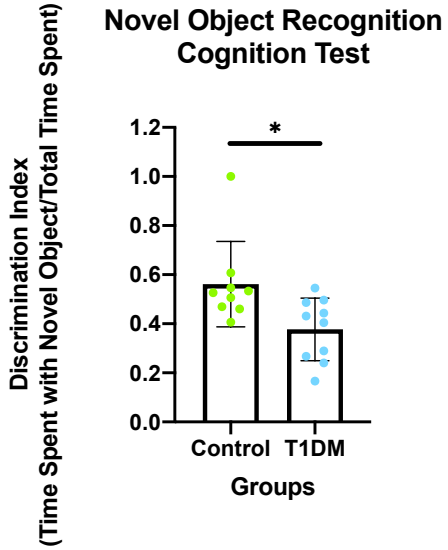


Figure 3.8: Novel Object Recognition Test Results

The graph shows the discrimination index calculated by the dividing time spent on the novel object by the total time spent of both objects. Each dot shows an animal in control and T1DM groups. There was a significance between control and T1DM values. Abbreviations: T1DM: Type 1 Diabetes Mellitus

3.3 Stainings

Hematoxylin and eosin staining was done on pancreas tissues of the mice to see the condition of pancreatic islets and islet cells. Compared to control group, T2DM mice pancreas tissue had bigger and swelled islets. T1DM mice pancreas islets were surrounded with immune cells (Figure 3.9).

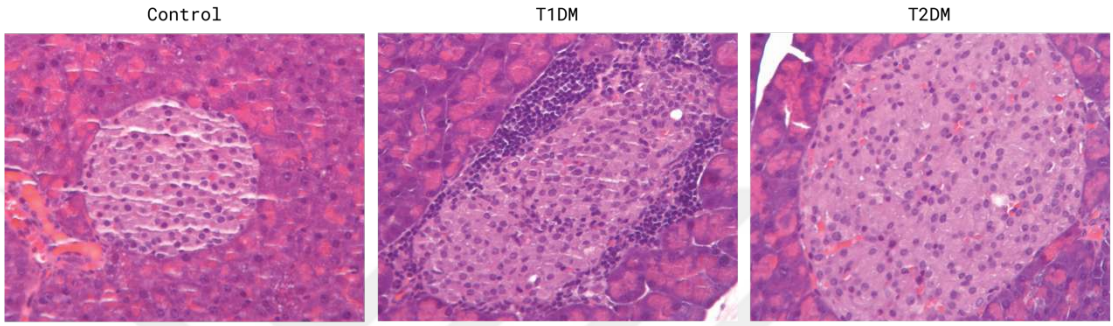


Figure 3.9: H&E Stainings of Control, T1DM and T2DM Mice Pancreas

The scale bar is 20μ. Abbreviations: T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

Immunohistochemistry staining against insulin was done to see the insulin secretion of the pancreatic islet cells of each group of mice. Compared to control mice, insulin seem to be less in T1DM mice. The islet cells were destroyed and immune cells were infiltrated in the tissue because of STZ injection and resulted in decreased insulin production. T2DM islet cells were swollen compared to control groups because of the excess insulin production (Figure 3.10).

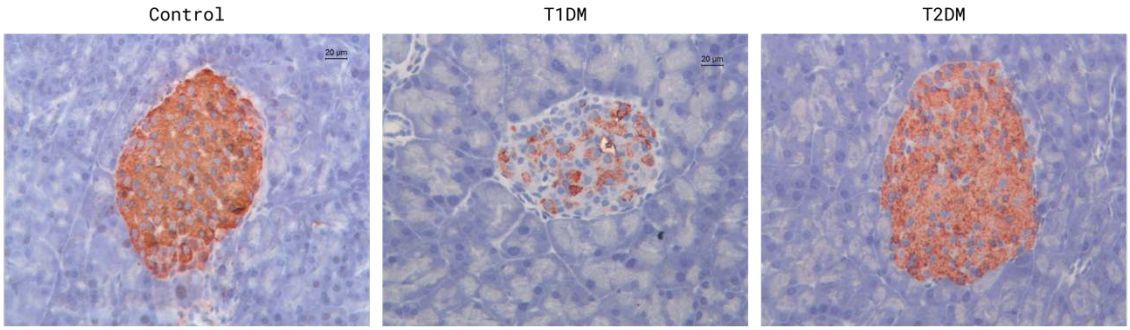


Figure 3.10: IHC Stainings of Insulin of Control, T1DM and T2DM mice

The scale bar is 20μ. Abbreviations: T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

Immunofluorescent stainings were done to see CD47 expression in the hippocampus area of the brain. The hippocampus sections of control, T1DM and T2DM were stained and different areas of hippocampus were visualised (Figure 3.13). The area stained with CD47, total cell area stained with DAPI and overlapped areas were analysed using ImageJ. CD47 stained area over total cell area and overlapped area over total cell area were calculated. CD47/DAPI results was significant (p= 0.0016) showing there is an increase in CD47 expression in T1DM and T2DM groups compared to control group (Figure 3.11). Similar to CD47/DAPI results, overlap area/DAPI results were also significant (p= 0.014) (Figure 3.12).

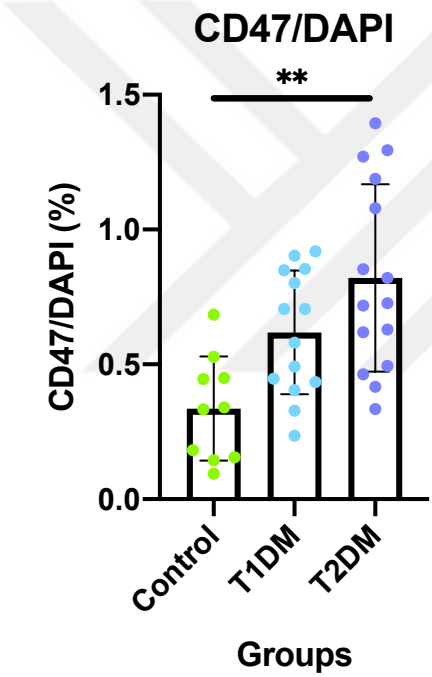


Figure 3.12: CD47 Stained Area over Total Cell Area

The graph shows CD47/DAPI area giving information about CD47 expressions. There is significant difference between groups. Abbreviations: T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

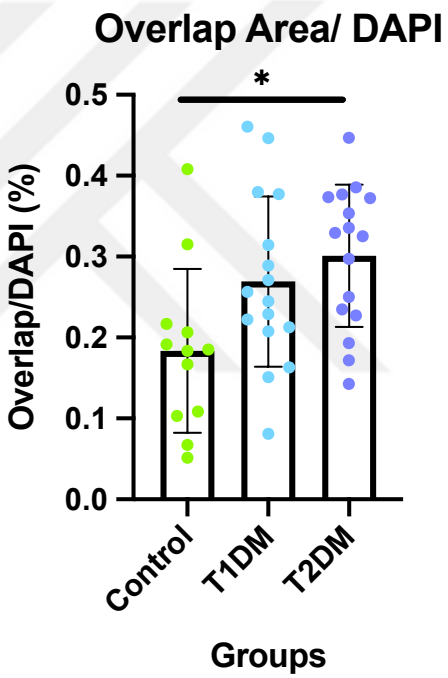


Figure 3.11: Overlap Area over Total Cell Area

The graph shows CD47 and DAPI/DAPI area giving information about CD47 expressions. There is significant difference between groups. Abbreviations: T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

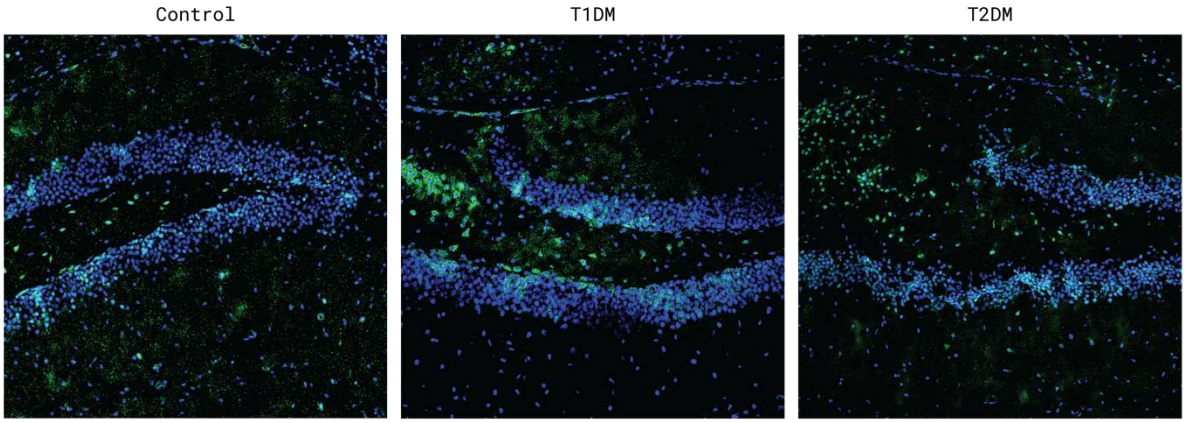


Figure 3.13: IF CD47 Stainings of coronal hippocampus sections of control, T1DM and T2DM mice

CD47 is stained with AF514, in green. DAPI is stained in blue. The scale bar is 75µ. Abbreviations: IF: Immunofluorescent; AF: Alexa Fluor; T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

GFAP polyclonal antibodies were used for immunofluorescent staining as a marker of astrocytes. Double stainings of CD47 and GFAP was done to check if astrocytes express CD47 (Figure 3.14). The overlapping images showed that some astrocytes express CD47.

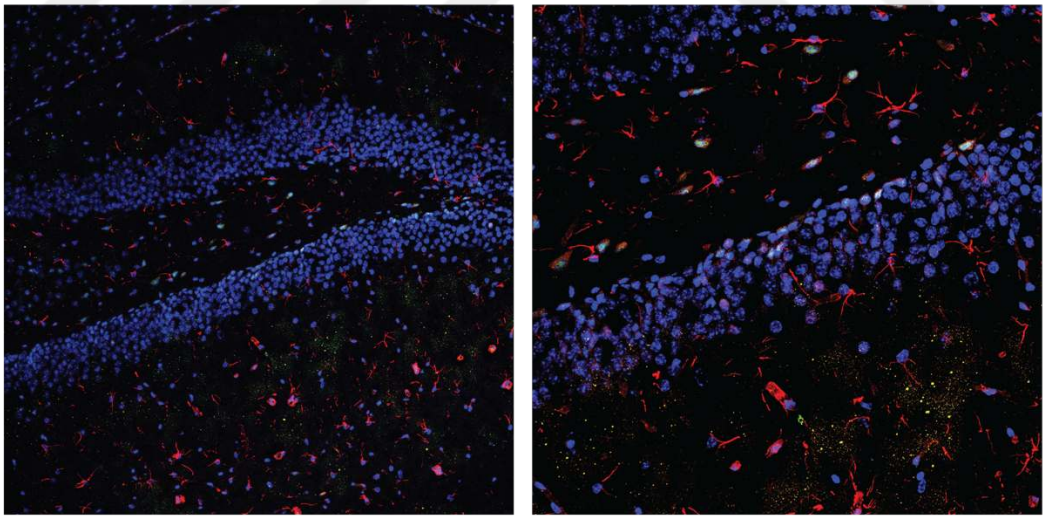


Figure 3.14: Double IF Staining of CD47 and GFAP

CD47 is stained with AF514, in green; GFAP is stained with AF 594 and DAPI is stained in blue. The scale bar is 75µ in the left figure and 120µ. Abbreviations: IF: Immunofluorescent; GFAP: Glial Acidic Fibrillary Protein; AF: Alexa Fluor; T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

NGF monoclonal antibodies were used as a marker of neurons. Double stainings of CD47 and NGF was done to check if neurons express CD47. CD47 and NGF stainings seemed to overlap showing that neurons express CD47 (Figure 3.15).

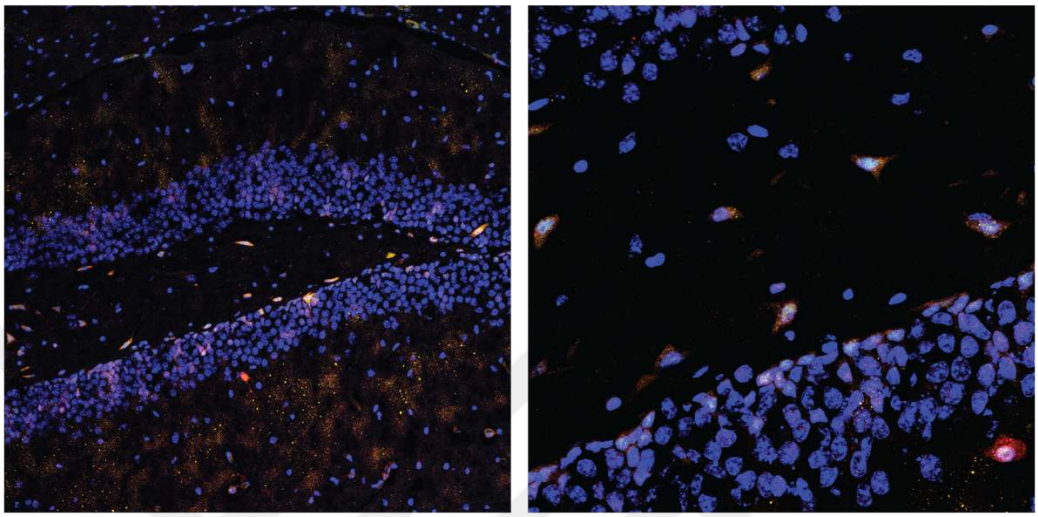


Figure 3.15: Double IF Staining of CD47 and NGF

CD47 is stained with AF514, in green; NGF is stained with AF 594 and DAPI is stained in blue. The scale bar is 75μ in the left figure and 150μ. Abbreviations: IF: Immunofluorescent; AF: Alexa Fluor; T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

IF Stainings of GFAP and NGF were also done with T1DM and T2DM groups to see the comparison of groups' expressions (Figure 3.16 & Figure 3.17). Although only one animal from each group was selected and their hippocampus were stained, the expression levels of GFAP seemed to differ among groups. Compared to control group GFAP expression seemed to increase in T2DM mice whereas it was decreased in T1DM mice (Figure 3.16). On the other hand, no big difference was seen in NGF expressions (Figure 3.17).

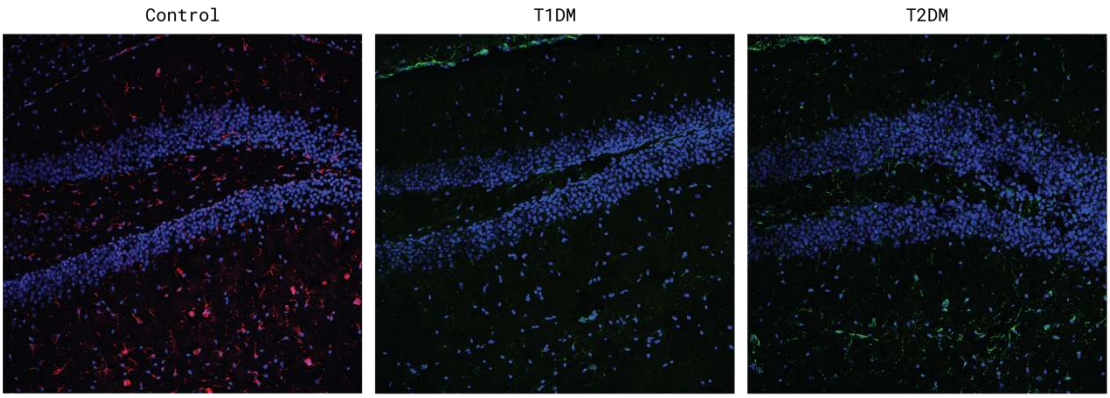


Figure 3.16: IF GFAP Stainings of coronal hippocampus sections of control, T1DM and T2DM mice

Control group is stained with GFAP with AF594, in red. T1DM and T2DM groups are stained with GFAP with AF514. DAPI is stained in blue. The scale bar is 75µ. Abbreviations: IF: Immunofluorescent; AF: Alexa Fluor; T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

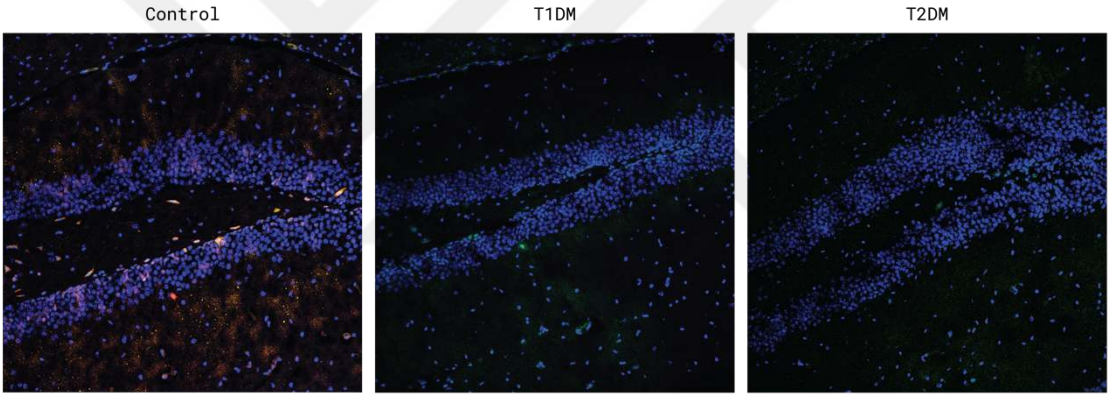


Figure 3.17: IF NGF Stainings of coronal hippocampus sections of control, T1DM and T2DM mice

Control group is stained with NGF with AF594, in red. T1DM and T2DM groups are stained with NGF with AF514. DAPI is stained in blue. The scale bar is 75µ. Abbreviations: IF: Immunofluorescent; AF: Alexa Fluor; T1DM: Type 1 Diabetes Mellitus; T2DM: Type 2 Diabetes Mellitus

3.4 Correlation

Table 3-1: Correlation between CD47 expression and Cognitive Functions

	Groups			CD47/DAPI	Overlap/DAPI
Spearman's rho	Control	OF (number of squares)	Correlation Coefficient	-0.6	-0.6
			Sig. (2-tailed)	0.4	0.4
			N	4	4
		Y-Maze (Alternation/Possible Rounds %)	Correlation Coefficient	0.6	0.6
			Sig. (2-tailed)	0.4	0.4
			N	4	4
		NOR (Discrimination Index)	Correlation Coefficient	0.8	0.8
			Sig. (2-tailed)	0.2	0.2
			N	4	4
	T1DM	OF (number of squares)	Correlation Coefficient	-0.3	-0.4
			Sig. (2-tailed)	0.624	0.505
			N	5	5
		Y-Maze (Alternation/Possible Rounds %)	Correlation Coefficient	0.3	-0.1
			Sig. (2-tailed)	0.624	0.873
			N	5	5
		NOR (Discrimination Index)	Correlation Coefficient	0.5	0.1
			Sig. (2-tailed)	0.391	0.873
			N	5	5

The table shows the correlation analysis results done with SPSS. Correlation is significant at 0.01 level.
Abbreviations: OF: Open Field; NOR: Novel Object Recognition

The result of Spearman's rho gave the correlation between CD47 expression and cognitive functions. Each test and their correlation with both CD47 expression data, CD47/DAPI and Overlap/DAPI, was analysed. Totally 12 correlation analysis was done and neither of the tests gave a r value close to -1.0 or 1.0. The r values for 12 tests are between -0.624 and 0.8. So, according to correlation analysis results, there is no significant correlation between CD47 expression and cognitive tests (Table 3-1).

Chapter 4:
DISCUSSION

2021 data of IDF suggests that 541 million people are in risk of diabetes and by 2030 the cases are thought to be 643 million and by 2045 the cases will rise to 783 million. The expenditure for this disease is assumed to be 966 billion dollars in 2021[IDF, 2021]. Hence, it can be seen that it is hard for government to support the patients and it is an economic burden for the patients. In addition, the disease affects many vital parts of the body and results in the death of the patient. Therefore, DM should be taken into serious consideration and more research should be done to extenuate the damage to the body.

One vital organ diabetes has an effect on is the brain. Although it is suggested that insulin resistance, excess glucose and inefficient insulin affects cognition and induce dementia by inducing an inflammatory response [Jellinger, 2015; Garcia Serrano, 2020]; the underlying molecular mechanism that dementia caused by diabetes is still to be discovered. CD47 is a surface protein having a role in the immune system homeostasis by working as a "Don't eat me!" signal. It has been showed by many research that CD47 is important for cognition and memory and is crucial for the development of neurodegenerative diseases [Junker, 2009; Gheibihayat, 2021]. There are also research showing the role of CD47 in DM in pancreatic tissue [Zhang, 2020] however, there is no work showing CD47 expression in DM models in the brain and whether it has a role in the cognitive impairment occurring because of diabetes. Therefore, in this research, investigating the correlation between CD47 levels and cognitive functions was aimed. To do this, diabetic mice models was constructed, CD47 expressions in the hippocampus area was checked using IF staining and cognitive functions were evaluated by three cognition tests. Correlation analysis was done lastly to investigate the relation between CD47 and cognitive functions.

39, 7-week-old CD1 male mice was used and grouped as control (n=12), T1DM (n=11) and T2DM (n=16). To induce T1DM model, low-dose STZ injection was done for five consecutive days. This method was used since it mimics human T1DM pathogenesis in the best way resulting in a partial and gradual islet cell destruction following an inflammatory process and a delayed hyperglycaemia [Wu, 2008]. As expected, T1DM mice first lost weight in response to STZ injection likewise to literature [Ho, 2011]. Their blood sugar increased in one week after STZ injection except three

mice. Anyhow, STZ is a chemical that is degraded instantaneously when dissolved in sodium citrate buffer therefore, injections should be done immediately after dissolving. Since there were many mice in the T1DM group, although the injections were done fast, STZ might be degraded leading to ineffective injection. This may be the explanation why three mice in T1DM group show a late response to STZ injections and resulted in low and different blood sugar levels after injections [Lee, 1993]. H&E stainings of T1DM pancreas showed the infiltration of immune cells to islet cells for their destruction. In addition, from IHC images it can be seen that, T1DM pancreas resulted in less insulin secretion. These findings are compatible with literature showing that T1DM islet cells are destroyed with STZ which mimics the destruction of islet cells with autoantibodies [Yi Lin, 2015].

In humans, an unhealthy diet and resulting obesity is the highest risk factor for insulin resistance and T2DM. Hence to induce T2DM model HF diet was used. In response to the HF diet, the mice started to gain weight significantly after week 5. Their blood sugar measurements were not high as expected however, OGTT results gave insights about the diabetic condition of the mice. H&E stainings together with IHC stainings of insulin of T2DM mice showed the swelled islets in the pancreas. The ß-cells were trying to produce more insulin as the adipose and muscle cells cannot utilize the circulating insulin because of the insulin resistance. These findings seem to be compatible with the existing results as well [Lin, 2019].

To evaluate the cognitive functions three cognition tests were performed: Open Field, Spontaneous Alternation in the Y-Maze and Novel Object Recognition. Unfortunately, T2DM mice started to die unexpectedly. Therefore, cognition tests could not be performed with T2DM mice; the tests were only done on T1DM group. Since the main purpose of this research was to investigate the CD47 and cognitive functions on both diabetic models, we want to perform these tests on T2DM groups as well in further experiments.

First test was the open field test. It is a sensorimotor test to determine general locomotor activity and to evaluate the responses to outer stimuli. In this test, the number of squares mice passed was counted to see the activity and basic movements of the mice. It was expected for control mice to have higher number of squares passed as a result of better basic movement and T1DM mice to pass through less squares because of cognitive impairment. As expected, control mice passed more squares than the T1DM mice

showing a better locomotor activity. These results were significant and showing compatible results with the existing research [Sharma, 2010].

Second test, which is Spontaneous Alternation in the Y-maze, is done to evaluate the cognitive deficits and to test spontaneous behaviour of the animal depending its memory. The spontaneous alternation percentage was calculated as stated in materials and methods and results parts. Since excess glucose and T2DM causes dementia, it was expected to see higher alternation in control mice than T2DM mice. However, on the contrary to OF and NOR tests, there was no significant difference between the groups. This was however compatible with some research done on db/db diabetic mice. They also suggest a non-significant result between control and diabetic mice in the Y-maze test [Sharma, 2010]. In addition, this test was the first test performed on the animals. Therefore, the data and the significance may be different from the other tests because of this. Although the animals got used to me, still being in a different environment may have resulted in stress. Because of the stress, the data may had given unassociated results.

Third and last test was the Novel Object Recognition test. It was done for evaluating the tendency of the animal to recognize a new object so the main aim is to assess the visual memory. The time spent on the new object is the primary measurement in this test. We were again expecting better visual and short term memory from control group. As expected, the results of the test showed increased discrimination index for control group. The difference between the groups were significant. The research done on sucrose-induced obese rats suggests a difference in NOR results [Jurdak, 2009] however, on the contrary, there are also research done on STZ administered diabetic rats showing no deficits in short term memory as a result of NOR test [Kassab, 2019].

To check the CD47 expression IF staining was done. After validating the DM models, the mice were sacrificed. Since the mice were sacrificed with exsanguination for further blood analysis and all of the organs are collected, perfusion could not be performed. There are evidences of doing perfusion fixing rather than immersion fixing gives higher quality of subjective histology [McFadden, 2019]. Therefore, this may have affected the quality of immunofluorescent staining. After collecting the animals, the organs were fixed in PFA for just one day to avoid overfixation and losing the epitopes needed for IF staining. The brains were further fixed in sucrose solutions with increasing concentration. After fixation, the tissues were stored in -80° in OCT. 10µm sections of brains were taken to perform IF staining. To show the CD47 expressions Primary Rabbit

Polyclonal anti-CD47 antibody was used together with AF514 secondary goat anti-rabbit antibody. The official Abcam page suggested that the quality of Primary Rabbit Polyclonal anti-CD47 antibodies, ab175488, did not meet the quality expectations by the users therefore, the distribution of the antibody was discontinued by Abcam. IF stainings were done using this antibody and it gave images with high background noise and non-specific interactions so the reason behind this may be due to the quality of the antibody. Also, poor washing of both the primary and secondary antibody results in high background however, the IF stainings were done with the optimized protocol specifically for the antibody.

CD47 is the receptor for SIRPα. Therefore, checking the SIRPα and its relationship with cognitive functions would also be the validation for CD47 expression and would give more insights. Taking this into consideration, IF staining was also thought to be done with Primary Rabbit Polyclonal anti- SIRPα antibody however, the primary antibody for SIRPα didn't work. Confocal images were full with background noise and non-specific interactions. $CD11c^+$ is the microglia marker. Since CD47 and SIRPα are immune system related proteins, microglias were wanted to be stained. However, the same problem SIRPα antibodies have, was valid for $CD11c^+$ antibody. So, the staining could not be performed.

To gather preliminary data and to validate the already existing data IF stainings of GFAP and NGF were also done. The main aim for GFAP staining was to check whether astrocytes are expressing CD47 or not in the control group. It can be seen from GFAP and CD47 double stainings that some astrocytes express CD47. In addition, one staining from T1DM and T2DM groups were also done to investigate the change in GFAP expressions. Comparing IF confocal images, T1DM showed decreased expression of GFAP unlike some works. In those research, the increase in GFAP expression was showed in STZ induced T1DM models suggesting activation of glial cells as in morphology and cell number in T1DM conditions [Nagayach, 2014]. Indeed, in the thesis research only one animal brain section is stained so, it is not conclusive. On the other hand, confocal images of T2DM mice hippocampus stained with GFAP showed an increase compared to control. Again, since there is only one sample stained it is hard to say something certain but the increase is compatible with the literature. Lizarbe et al.

showed the increase in immunoreactivity against GFAP suggesting an increase in gliosis in T2DM mice induced with HF diet [Lizarbe, 2019].

The main aim for NGF staining also was to check whether neurons are expressing CD47 or not in the control group. It can be seen from NGF and CD47 double stainings that some neurons express CD47. In addition, one staining from T1DM and T2DM groups were also done to investigate the change in NGF expressions. Comparing IF confocal images, no big difference was seen in NGF expressions between groups. Indeed, only one animal brain section is stained so, nothing obvious was expected and this is not conclusive. Neuropathy research suggest that there is a decrease in NGF expression in diabetic neuropathy. It is believed that the decrease in NGF can be due to glucose-induced oxidative stress or immune neutralization as a result of autoimmunity [Pittenger, 2003]. Therefore, in the light of this knowledge it is expected to see a decrease in NGF in diabetic models. For further experiments NGF expression together with CD47 expression should be taken into consideration.

For both GFAP and NGF, there are also scientific work showing their relation with cognition and neurodegenerative diseases [Jönhagen, 2000; Yang, 2015].

This research has showed that there is an increase in CD47 expression in the hippocampus region of the brains of T1DM and T2DM models. There was also a significant difference in two out of three cognition tests between control and T1DM models. However, the correlation analysis results showed that there is no correlation between CD47 expressions and cognitive functions. Therefore, the increase in CD47 levels can be explained as the cells' way of protecting themselves. An increase in CD47 expression of cancer cells were explained as their way of escaping from phagocytosis of immune cells [Jaiswal, 2019]. This increase in CD47 expression can also be a route of circumventing the negative effects of extra glucose initiated inflammatory response.

I believe that this experiment has enlightened the way for future experiments. This is the beginning for CD47, cognition and DM research. In T2DM treatment, a medication called Metformin is used to regulate the blood sugar. It works through reducing the glucose levels released into the blood by the liver, makes the cells respond better to insulin [Hundal, 2000]. There are some scientific work suggesting that Metformin not only regulates the blood sugar but also has an effect on cognition and cancer. DiBona et al. showed in their research that metformin has an effect on cognitive functions, spatial learning and nest building, in mice undergoing controlled cortical impact injury [DiBona,

2021]. So, it may be a promising medication not only for DM but also neurodegenerative diseases. Human studies aiming to study the effects of metformin on cognitive functions are existing. The results have no significance at the moment but they suggest to do further work to conclude the specific effects [Campbell, 2018; Ng, 2014; Koo, 2019]. There are also some work present suggesting metformin usage reduces the risk of diabetic dementia [Chin-Hsiao, 2019]. Out of many research, Tan et al. has a research showing the effect of Metformin on breast cancer stem cells (BCSC). Their work suggests that Metformin uses CD47 and miR-708 to inhibit the self-renewal of BCSCs [Tan, 2018]. Therefore, based on this knowledge showing the cognitive effects and showing the target of Metformin as CD47, our aim for the future is to conduct the same research with Metformin treated mice. I hope that this future research will give many more insights.

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Appendix A:

Table 0-1: Standard Mice Chow Contents for Control and T1DM Mice

Kliba Nafag		
3430		
Mouse & Rat		**Maintenance**
Standard		
Major Nutrients		**Ingredients**
Dry Matter	88.00%	Wheat, barley, soybean meal (NGMO), wheat middlings, corn (NGMO), poultry meal, wheat starch, whey powder, soybean oil, oats, brewer's dried yeast, minerals, vitamins, amino acids
Crude Protein	18.50%	
Crude Fat	4.50%	
Crude Fibre	4.50%	
Crude Ash	6.30%	
NFE	54.20%	
Gross Energy	16.1 MJ/kg	
Metbol. Energy	13.2 MJ/kg	
Starch	35.00%	
Amino Acids		
Arginine	1.10%	
Lysine	1.00%	
Methionine	0.39%	
Methionine + Cystein	0.75%	
Tryptophan	0.20%	
Threonine	0.65%	
Major Mineral Elements		**Remarks**
Calcium	1.05%	Complete food for mice and rats, also appropriate for hamsters and gerbils Given values are calculated averages in air-dry feed
Phosphorus	0.80%	
Magnesium	0.20%	
Sodium	0.20%	
Potassium	0.78%	
Chlorine	0.36%	
Trace Elements		
Iron	290 mg/kg	
Zinc	60 mg/kg	
Copper	14 mg/kg	
Iodine	1 mg/kg	
Manganese	60 mg/kg	
Selenium	0.3 mg/kg	

Vitamins		**Delivery form**
Vitamin A	15'000 IU/kg	Pellets 12 mm round 15 kg in paper bags
Vitamin D3	800 IU/kg	
Vitamin E	102 mg/kg	
Vitamin K3	4 mg/kg	
Vitamin B1	27 mg/kg	
Vitamin B2	15 mg/kg	
Vitamin B6	13 mg/kg	
Vitamin B12	0.07 mg/kg	
Nicotinic Acid	90 mg/kg	
Pantothenic Acid	41 mg/kg	
Folic Acid	3 mg/kg	
Biotin	0.3 mg/kg	
Choline	2'000 mg/kg	
Vitamin C	36 mg/kg	

Table 0-2: High Fat Mice Chow Contents for T2DM Mice

Kliba Nafag		
2127		
Mouse & Rat		**Experimental Diet, Purified Diet**
High Fat Diet: 60 % kcal % fat		
Major Nutrients		**Ingredients**
Dry Matter	92.00%	Lard, casein, maltodextrin, sucrose, cellulose, refined soybean oil, minerals, vitamins, amino acids
Crude Protein	23.90%	
Crude Fat	35.00%	
Crude Fibre	4.90%	
Crude Ash	5.00%	
NFE	23.20%	
Gross Energy	23.8 MJ/kg	
Metbol. Energy	22.0 MJ/kg	
Starch	1.00%	
Amino Acids		
Arginine	0.93%	
Lysine	1.62%	
Methionine	0.54%	
Methionine + Cystein	0.98%	
Tryptophan	0.39%	

Threonine	0.93%	
Major Mineral Elements		**Remarks**
Calcium	0.83%	Experimental diet for mice and rats Given values are calculated averages in air-dry feed Production on demand
Phosphorus	0.50%	
Magnesium	0.08%	
Sodium	0.27%	
Potassium	0.57%	
Chlorine	0.21%	
Trace Elements		
Iron	80 mg/kg	
Zinc	48 mg/kg	
Copper	8 mg/kg	
Iodine	0.6 mg/kg	
Manganese	14 mg/kg	
Selenium	0.2 mg/kg	
Vitamins		**Delivery form**
Vitamin A	10'400 IU/kg	Pellets 10 mm round 5 kg in welded aluminium bag
Vitamin D3	1'040 IU/kg	
Vitamin E	357 mg/kg	
Vitamin K3	6 mg/kg	
Vitamin B1	23 mg/kg	
Vitamin B2	15 mg/kg	
Vitamin B6	11 mg/kg	
Vitamin B12	0.05 mg/kg	
Nicotinic Acid	47 mg/kg	
Pantothenic Acid	39 mg/kg	
Folic Acid	2 mg/kg	
Biotin	0.2 mg/kg	
Choline	1'350 mg/kg	