Nagore Izal Razquin

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EFFECT OF A HIGH FAT DIET IN THE EXPRESSION OF ALZHEIMER´S DISEASE AND TYPE 2 DIABETES MELLITUS RELATED miRNAs IN APPswe/PS1dE9 MICE CORTEX TISSUE

Academic tutor: Ricardo Rodríguez Calvo
In cooperation with: Nutrition and Metabolic Health research group

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1 ABSTRACT

BACKGROUND: Type 2 diabetes mellitus (DMT2) is a metabolic condition characterized by insulin resistance (IR), in which the body's cells do not adequately respond to this hormone, leading to chronic hyperglycemia. IR in the brain has been found to be associated with a faster progression of Alzheimer's disease (AD), which is the most common cause of dementia, suggesting that AD can be considered a metabolic disease. This accelerated progression of the disease has been observed to be due to the dysfunction of processes such as synaptic function, memory and cognition. On the other hand, miRNAs have been shown to play a role in several cellular and physiological processes and their dysregulation has been linked to numerous diseases, including T2DM and AD. Furthermore, some studies have observed that diet can modify the expression of certain miRNAs in response to specific dietary bioactive agents.

HYPOTHESIS: The expression of insulin and AD related miRNAs in cortex tissue of mice is altered due to the influence of a high fat diet (HFD), which exacerbates both metabolic health issues and the progression of AD.

MATERIALS AND METHODS: Samples of cortex were obtained from a total of 56 male mice of the C57BL/6J strain, which were 6 months old. These animals were divided into mice with the APPswe/PS1dE9 genotype, which are an animal model of AD, and mice with the WT genotype. Each different mice genotype was divided into two study groups, one fed with regular control (CT) diet and the other one fed with a HFD. Finally, gene expression was determined from tissue samples of selected miRNAs, which are involved in AD and T2DM. These miRNAs are miR146a-5p, miR155-5p, miR181c-5p, miR19a-3p, miR22-3p, miR29c-3p and miR320a-3p.

KEYWORDS: AD, T2DM, IR, miRNAs, cortex tissue, Aβ protein, HFD, mice, APPswe/PS1dE9, wild type.

2 ABBREVIATIONS AND ACRONYMS

A

AD: Alzheimer´s disease

ApoE4: Apolipoprotein E gene allele 4

APP: Amyloid precursor protein

Aβ: Beta amyloid

C

CNS: Central nervous system

Ct: Cycle threshold

D

DM: Diabetes mellitus

G

GLUT4: Glucose-transporter 4

H

HF: High fat

HFD: High-fat diet

I

IR: Insulin resistance

P

PCR: Polymerase chain reaction

PS1: Presenilin 1

PS2: Presenilin 2

T

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

W

WT: Wild-type

3 INTRODUCTION

3.1 DIABETES MELLITUS

Diabetes mellitus (DM) is the most common metabolic disorder, which is characterized by chronic hyperglycemia. In 2021 this disease affected more than 537 million people worldwide according to the International Diabetes Federation, meaning that more than 1 out of 10 people suffers from this disease [1].

There are two main forms of DM. Type 1 diabetes mellitus (T1DM), which represents the 10% of the diabetes cases, is caused by an autoimmune reaction where the body destroys pancreatic β cells, leading to a progressive loss of insulin secretion [2]. Type 2 diabetes mellitus (T2DM), which causes the 90% of the cases, is characterized by insulin resistance (IR) in target organs. The global increase of sedentary lifestyles, high caloric diets and obese and ageing people are important factors that have contributed to the increase in the prevalence of T2DM in recent years [3].

The development of T2DM involves various organs such as the pancreas, liver, skeletal muscle, kidneys, brain, small intestine and adipose tissue. Recent studies indicate that dysregulation of adipokines, inflammation, abnormalities in gut microbiota, immune dysregulation and inflammation have emerged as significant pathophysiological factors in T2DM [4].

INSULIN AND IR

Insulin is an anabolic hormone synthetized as a 51-amino acid peptide in the β-cells of the islets of Langerhans, in the pancreas, that plays a range of roles in peripheral tissues. Insulin helps to the capitation of glucose into cells, decreasing blood glucose levels, and stimulates the synthesis of glycogen and the capitation and storage of fat in adipose tissue. Also stimulates cell growth, differentiation and inhibits catabolic processes (glycolysis, lipolysis and proteolysis) [5].

IR refers to a decreased ability of target tissues, mainly the liver, muscle and adipose tissue to respond to insulin signals, which are essential for glucose regulation in the body. This leads to a reduced ability to dispose of glucose, forcing beta cells in the pancreas to produce more insulin to compensate, resulting in high levels of insulin in the bloodstream (hyperinsulinemia) [6].

The underlying molecular mechanism for IR is not fully understood, but several factors have been implicated in its development. One factor is polymorphisms in genes related to insulin signaling cascade; genetic variations in these genes can affect the expression, function and

regulation of proteins involved in insulin signaling, leading to impaired insulin action [7]. Another factor is post-translational modifications of proteins involved in insulin signaling, these post-translational modifications, such as phosphorylation, acetylation and/or glycosylation, can alter protein structure and function, affecting insulin signaling and glucose metabolism [8]. Protein degradation of components of the insulin signaling cascade is also implicated in IR, in this case, abnormal protein degradation, such as excessive degradation of insulin receptor substrate-1 (IRS-1), can impair insulin signaling [9]. There are other factors that also can contribute to IR including chronic inflammation, oxidative stress and lipotoxicity [10].

Focusing on the brain, the development of IR in this organ has been shown to play an important role in the pathogenesis of Alzheimer's disease (AD), hypothesizing that this disease may be considered as a metabolic disease [11].

In this organ, insulin regulates the expression of glucose-transporter 4 (GLUT4), which is crucial for the brain's metabolism of glucose, as it is responsible for the uptake of glucose into the cells and some studies have revealed reduced levels of GLUT4 expression in the brains of individuals with AD after death. Also, insulin have been related to facilitate the growth, metabolism, survival, gene activity and protein production of neurons and glial cells and also play a role in neurotransmitter signaling and synaptic function [11]. Therefore, the AD neurodegeneration is facilitated by the suppression of insulin signaling due to the increase of the activity of kinases that hyperphosphorylate tau, the accumulation of beta amyloid (Aβ) protein, oxidative and endoplasmic reticulum stress, generation of reactive oxygen and reactive nitrogen species that damage proteins, RNA, DNA and lipids, mitochondrial dysfunction, etc. IR also causes the down-regulation of target genes that are involved in cholinergic function, causing the dysfunction of neuronal plasticity, memory and cognition [12].

In fact, it has been shown that the administration of a HFD, which causes an obese phenotype, hyperglycemia, inflammation, oxidative stress and IR in mice [13], increases the accumulation of amyloid plaques, hyperphosphorylation of tau in the brain and a decrease in the cognitive function in APPswe/PS1dE9 mice, leading to an important affection in neuronal processes such as synapses, despite the mechanism by which this occurs is still unclear [14]. In addition, it has also been observed that this same diet also affects brain synaptic plasticity in mice that were not genetically predisposed to this disease due to an alteration in insulin-sensitive processes, which are essential for neuronal survival, learning and memory, processes that are related to the development of AD [15].

3.2 ALZHEIMER´S DISEASE

AD is a slowly progressive neurodegenerative disease that usually occurs in old people that affects around 50 million people worldwide, being the most common cause of dementia. In fact, it is believed that by the year 2050 the number of people affected will increase to reach 152 million [16]. AD often leads in the loss of the autonomy of the person who suffers from it, as it causes memory impairment, language impairment, personality changes and decline in thinking [17].

Regarding the symptoms of the disease, among them stands out memory loss, which is a key symptom that appears as a difficulty in remembering recent events or conversations. This symptom worsens as the disease progresses, eventually leading to problems identifying objects, people, expressing feelings, or even participating in conversations. Additionally, this disease also causes difficulty in decision-making and reasoning, as well as changes in the patient's personality and behavior (depression, apathy, mood swings, irritability, aggression, etc.) [18].

A small fraction (approximately 2%) of early-onset familial AD can be attributed to genetic mutations in amyloid precursor protein (APP), presenilin1 (PS1) and presenilin 2 (PS2). In addition, late-onset AD is linked to certain genetic risk factors such as the apolipoprotein E gene allele 4 (ApoE4) genotype and a variant in the sortilin-related receptor 1 gene. However, recent studies suggest that environmental factors and dietary habits are also crucial contributors to the development of late-onset AD [19].

This disease is characterized by the loss of neurons and synapsis and the presence neuroinflammation, senile plaques and neurofibrillary tangles [20], which mostly affect the medial temporal lobe, where the hippocampus is located, and the cerebral cortex [21]. These two brain structures have been recognized as the most important structures related to amnestic processes. Specifically, the hippocampus has been associated with the functions of consolidation of recently acquired information, which involves the short-term memory (minutes, hours or a few days), while the cortex has been found to be related to long-term memory [22].

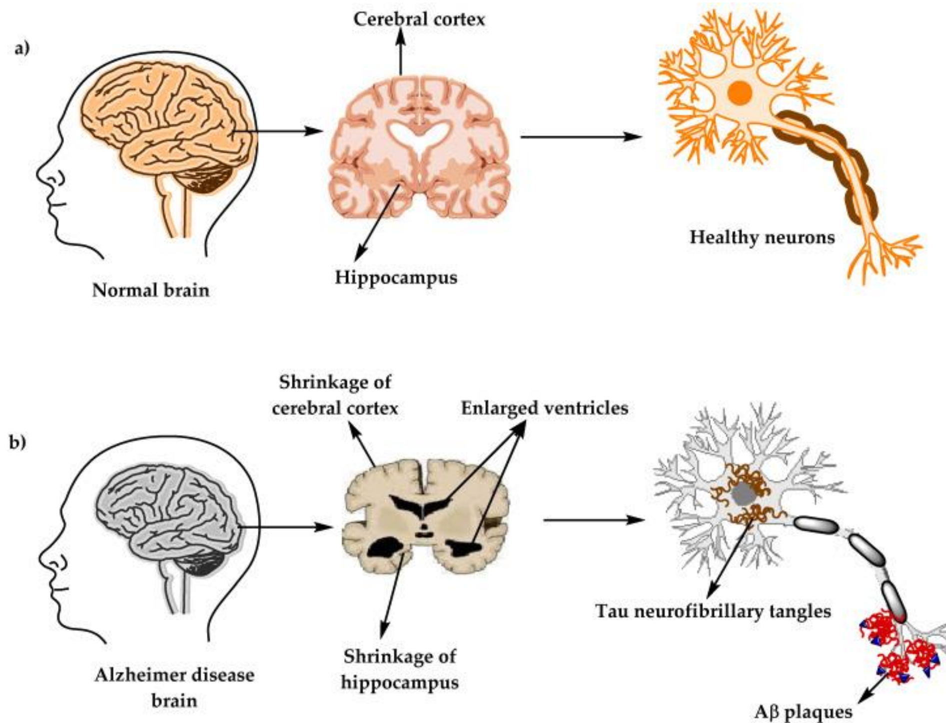

Figure 1: a comparison of the brain structure in a person without (**a**) and with (**b**) AD [23].

Senile plaques are extracellular accumulations of Aβ that are synthetized by β-secretases and γ-secretases by proteolytic cleavage of the amyloid precursor protein (APP) [23]. This APP is a type-1 transmembrane integral glycoprotein that is one of the most abundant proteins in the central nervous system (CNS) [21]. The β-secretases and γ-secretases cleave APP leading to several Aβ protein species, depending on the number and sequence of amino acids. In the brain, the species with 40 and 42 amino acids are the most abundant (Aβ40 and Aβ42 respectively) [24].

The mechanism by which Aβ accumulation causes cellular damage is still not clear, but several ways have been proposed through which Aβ accumulation could damage neurons: by activating the microglia, which is the innate immune system in the central nervous system, triggering the inflammatory response and the release of neurotoxic cytokines, causing oxidative damage, inducing apoptosis mechanisms, impairing perfusion due to the accumulation of amyloid in capillaries and arterioles and affecting interneuronal synaptic contacts [25].

Neurofibrillary tangles are an unusual accumulation of a protein called tau that develops within neurons. Neurons rely on internal structures known as microtubules to support their functioning, which aids the transport of essential nutrients and molecules from the cell body to the dendrites and axon. Under normal conditions, tau binds to microtubules and stabilizes them. However, in AD, tau becomes chemically altered, causing it to detach from microtubules and

clump together with other tau molecules, creating threads that ultimately combine to form tangles within neurons. As a result, the transport system within neurons becomes blocked, causing a breakdown in synaptic communication between neurons [26].

3.3 MIRNAS

miRNAs, or microRNAs, are non-coding ARN molecules, usually with a length of 21-23 nucleotides, that regulate gene expression normally by post-transcriptional regulation [27]. These oligonucleotides only represent the 2-3% of the human genome, but It is thought that the expression of approximately 60% of all genes is regulated by these molecules [28]. One miRNA can regulate approximately 200 different mRNAs involved in different cellular pathways, but one mRNA can be regulated by multiple miRNAs [29, 30].

The regulation mostly occurs when miRNAs interact with the complementary sequence of the 3´ UTR of target mRNAs, normally causing a suppressed expression or degradation, mainly by cleavage, but it also exists miRNAs with promoter or coactivator action [31, 32]. The mechanism of the repression is still poorly understood but it appears to be involved in the inhibition of the mRNA synthesis initiation [33]. However, other type of interactions between miRNAs and other regions of mRNAs have also been described, including the interaction with the 5´ UTR, coding sequences and gen promoters [34].

miRNAs have been demonstrated to be involved in several cellular and physiological processes, including cell differentiation, proliferation, apoptosis, morphogenesis, fat metabolism, hormone secretion and the formation of long-term memory. In fact, the dysregulation of miRNAs has been linked to numerous diseases, including various types of human cancer, T2DM, as well as cardiovascular, skeletal muscle and neurodegenerative disorders such as AD and amyotrophic lateral sclerosis [35, 36]. For example, in previously conducted in vitro and in vivo studies using different AD models, it has been observed that this microRNA is associated with Aβ deposition and pathological changes in synapses [37]. Additionally, in a mice model of AD, it was demonstrated that this microRNA was significantly overexpressed compared to the control group (mice without AD). Furthermore, in the same study, it was also observed that miR-146a-5p promotes increased Aβ accumulation through the generation of ROS, leading to oxidative stress and promoting neuroinflammation [38].

In addition, so many studies have shown that diet can modify the expression of certain miRNAs. It has been proved that some miRNA expression changes in response to certain dietary bioactive agents, including PUFAs, vitamins and phytochemicals [39]. For example, a study carried out in mouse liver found that the levels of miR-27b were notably induced in response to a high-fat diet (HFD) that provided 42% of calories. This is a miRNA that regulates lipid metabolism by modulating genes such as Angptl3, which plays a crucial role in triacylglycerol metabolism [40].

4 HYPOTHESIS AND OBJECTIVES

The hypothesis of our project is that a HFD, which aggravates both the metabolic health and the progression to AD, alters the expression of insulin and AD related miRNAs in the cortex tissue of mice. This could help to explain the more rapid development of AD in APPswe/PS1dE9 mice, which is an animal model of AD, fed with a HFD and the alteration of synaptic processes in wild type (WT) mice fed with this same diet.

As for the objectives, these are to analyze if any of these miRNAs alter their expression in the cortex tissue of a mice model of this disease comparing with WT mice and to observe if a HFD can modulate the expression of these miRNAs in the same tissue and genotype of animals.

5 MATERIALS AND METHODS

5.1 ANIMALS, DIET AND SAMPLES

ANIMALS

The present study has been performed using a total of 56 6-month-old male mice of the C57BL/6J strain. These animals were divided into mice with the APPswe/PS1dE9 genotype, which have been defined as a model of AD, and mice with the WT genotype, which are not genetically predisposed to suffer from this disease.

The double transgenic APPswe/PS1dE9 mice co-express a Swedish (K594M/N595L) mutation of a chimeric mouse/human APP (Mo/HuAPP695swe), in addition to the exon-9-deleted variant of presenilin-1 gene (PS1-dE9) [41]. These two mutations have shown to be related in pathological changes similar to those observed in AD, including severe age-dependent neuropathology and global brain atrophy. In addition, APPswe/PS1dE9 mice also show deficits in working memory in the early phase of the disease, which is similar to humans with AD. Moreover, in the later phase of the disease the mice also exhibit deficits in spatial memory and taste aversion learning. Additionally, non-cognitive related symptoms associated with AD, such as irritability, disturbances in the diurnal cycle and motor functions, depression and anxiety also appear [42]. In fact, in a previous study carried out by the collaboration of the Nutrition and Metabolic Health research group (NuMeH), it was observed that mice of the same strain, genotype (APPswe/PS1dE9) and age used in this study exhibited memory loss when Novel object recognition test carried out [43].

The animals in this study were housed under standard conditions with a 12-hour light-dark cycle and provided with free access to food and water. All animal procedures were carried out in accordance with ethical guidelines outlined in the European Communities Council Directive 2010/63/EU and approved by the local ethical committee (UB). Also, the researchers made every effort to minimize animal suffering and reduce the number of animals used in the study.

DIET

Each different mice genotype was divided into two study groups, one fed with regular control (CT) diet and the other one fed with a HFD, with a total of 14 mice in each of the 4 study groups. Both CT diet and HFD were administered to the animals from weaning, 21 days after birth, until they were sacrificed at 6 months old.

The HFD used in this study was obtained from Research Diets, Inc (D12492 formula), whose composition consists mostly of fat, providing 60% of the total energy intake compared to the 18% of the total energy intake provided by fat in the CT diet. These diet's composition is shown in **Table 1**.

Table 1. Description of the diet composition and caloric content of the CT and HF diets.

	CT diet	*HFD*
	Kcal %	***Kcal %***
Protein	24,0	20,1
Carbohydrate	58,0	19,7
Fat	18,0	60,0
Total	100,0	100,0
Ingredients		***g***
Casein, Lactic, 30 Mesh		200
Cystine, L		3
Lodex 10		125
Sucrose, Fine Granulated		72,8
Solka Floc, FCC200 (fiber)		50
Lard fat		245
Soybean Oil, USP		25
Mineral Mix S10026B		50
Choline Bitartrate, V10001C (vitamins)		3
Dye, Blue FD &C #1, Alum. Lake 35-42%		0,05
Total		773,85

SAMPLES

Due to its direct involvement in AD and in order to understand more about how this disease affects the brain, cortex tissue samples were selected to perform the study.

This samples were obtained, frozen and stored at -80ºC until further processing immediately after the animals were sacrificed.

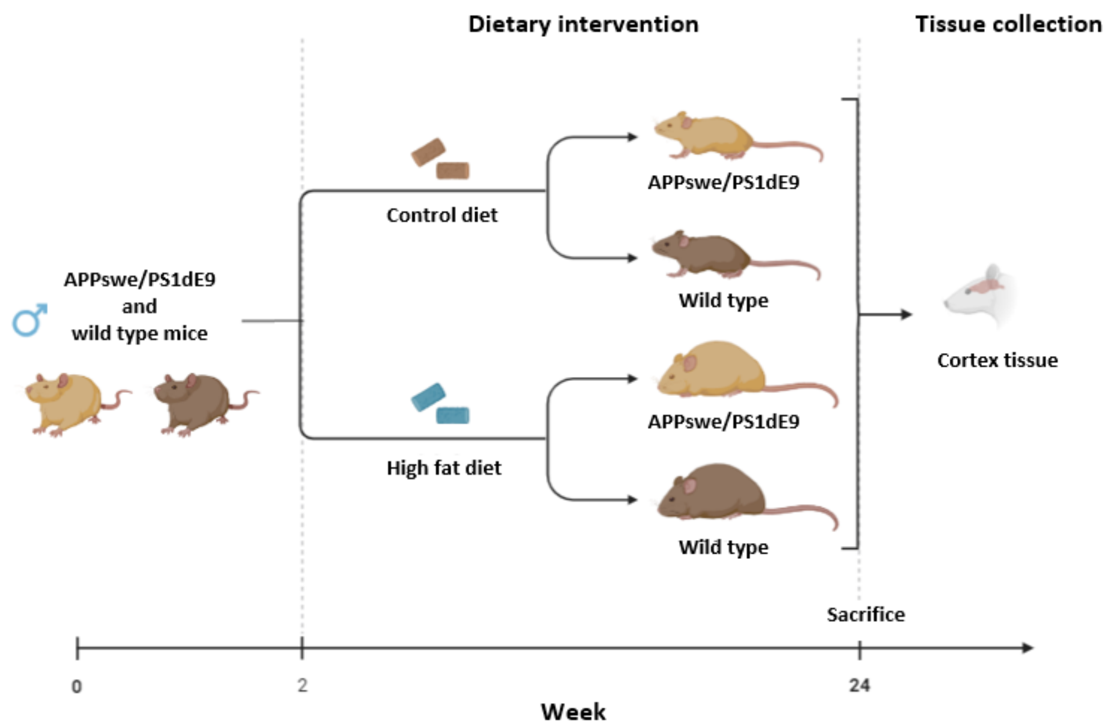

Figure 2. Summary of the procedure performed in the diet intervention and tissue collection in each mice genotype. (Created with Biorender).

5.2 MIRNAS

miRNA SELECTION

To help test our hypothesis, the selected miRNAs in the study were those that, according to the current existing literature, have been found to be associated with AD and T2DM. These miRNAs are miR146a-5p, miR155-5p, miR181c-5p, miR19a-3p, miR22-3p, miR29c-3p and miR320a-3p. The details of this association are shown in **Table 2**.

Table 2. Relationship between miRNAs 146a-5p, 155-5p, 181c-5p, 19a-3p, 22-3p, 29c-3p and 320a-3p, AD and T2DM.

miRNA	*AD*	*T2DM*
miR146a-5p	Related to synapse formation and neuronal development. Overexpressed in the brain of rats with AD, causing neuroinflammation and neurodegeneration [44].	Controls the expression of genes implicated in the development of T2DM and its associated complications [45]. It has been observed to be downregulated in patients with this disease, leading to inflammation and IR [46].
miR155-5p	Increases Aβ deposition and expression of proteins related to Aβ generation, such as β-secretase and γ-secretase. Its inhibition improved cognitive impairment, neuronal regeneration and decreased Aβ deposition [47].	Participates in the pathogenesis of diabetic complications, such as nephropathy, neuropathy and cardiomyopathy [48]. Expression levels of this miRNA have been found to be repressed in serum from diabetic patients. [49].
miR181c-5p	It has been associated with an increase in Aβ generation by promoting the inhibition of γ-secretase in AD pacients [50].	In the diabetic db/db mice model it was observed that this miRNA was repressed in aortic endothelial cells. [51].
miR19a-3p	Its expression has been observed altered in plasma and brain of patients with AD [52, 53].	Promotes de proliferation and insulin secretion. Also inhibits the apoptosis of pancreatic β cells. It has been observed repressed in patients with T2DM [54].

miR22-3p	MiR-22-3p overexpression reduced Aβ deposit and ameliorated AD symptoms in a mice model of this disease [55].	Its inhibition has reduced fat accumulation, circulating glucose levels, cholesterol levels and improved insulin sensitivity related to T2DM in mice and in primary cultures of human subcutaneous adipocytes [56, 57].
miR29c-3p	The expression levels of this miRNA have been observed to be repressed in the brain of AD mice. It is thought that it may be involved in the reduction of cytotoxicity caused by Aβ accumulation [58].	Has been found to be overexpressed in various tissues and cell types in conditions of metabolic disease, obesity, IR and T2DM [59]. Is overexpressed in skeletal muscle of T2DM mice [60].
miR320a-3p	It has been observed repressed in plasma of patients with AD. [61].	The overexpression increases oxidative stress in β cells, resulting in decreased proliferation, increased apoptosis and subsequently reduced insulin secretion [62].

In addition, to make sure the results were not altered by external factors, negative and positive controls were also analyzed. A miRNA from *Caenorhabditis elegans*, cel-miR-39-3p, was used as a positive control and another miRNA from *Arabidopsis thaliana*, ath-miR-159a, was used as a negative control.

miRNA OBTAINING

RNA EXTRACTION AND cDNA SYNTHESIS

The cortex samples were homogenized using the IKA ULTRA-TURRAX T 18 Digital Disperser (Thermo Fisher Scientific, USA) before the processing, also the exogenous miRNA, or spike in, from *Caenorhabditis elegans* was added considering a proportion of 3:200 for all the samples. Then, total RNA extraction was performed using the Invitrogen mirVana PARIS RNA and Native Protein Purification Kit (Thermo Fisher Scientific, USA). The concentration and purity of the total RNA were controlled by using the NanoDropTM 1000 Spectrophotometer (Thermo Fisher Scientific, USA), which is a spectral scanning UV-VIS spectrophotometer to measure the concentration and quality ratios of the samples.

Once total RNA was obtained, Applied Biosystems TaqMan Advanced miRNA cDNA Synthesis *Kit* (Thermo Fisher Scientific, USA) was used to obtain the corresponding cDNA samples necessary to perform the qPCR.

qPCR

The protocol used to perform the quantitative polymerase chain reaction (PCR) was the Applied Biosystems TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, USA), using the specific primers of the miRNAs selected plus the *Caenorhabditis elegans* and *Arabidopsis thaliana* miRNAs, which were all supplied by the commercial company itself. Once the 396-well plates were prepared with this protocol, the Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA) was configured to run the PCR applying the time and temperature cycles shown in **Table 3**.

Table 3. Number of cycles, temperature and time used in each stage of the qPCR process.

Step	*Temperature*	*Time*	*Cycles*
Enzyme activation	95 °C	20s	1
Denature	95 °C	1s	40
Anneal / Extend	60 °C	20s	

From the PCR, cycle threshold (Ct) values were obtained, which were used for the statistical analysis. These values indicate the number of amplification cycles necessary for the fluorescent signal generated by the increase in the amount of PCR products to reach a detectable level.

5.3 STATISTICAL ANALYSIS

Two different types of analysis were performed, the analysis of comparison by diet (the APPswe/PS1dE9 - CT vs the APPswe/PS1dE9 - HF and WT-CT vs WT-HF) to see the effect of a HFD on the expression of these miRNAs in each genotype and the analysis of comparison by genotype (the APPswe/PS1dE9 CT vs WT CT) to see the effect of each genotype on the expression of these miRNAs.

Through the Ct values obtained in the q-PCR, the 2^-ΔΔCT method was performed, which is used to calculate relative changes in gene expression, in both analyses independently. This method is used to obtain the fold change value, which indicates the degree of change in expression. A fold change value equal to 1 means that there is no change, values less than 1 means that there is a repression and a value greater than 1 means that there is an overexpression of the gene. Outliers (abnormal dCt values) were discarded using the z score method, in which samples with z score values greater than +2,5 and less than -2,5 were eliminated.

SPSS software was used to plot the logarithm in base 10 of the fold change values obtained for each miRNA in both analyses.

Finally, normality tests were also performed in SPSS software to determine the type of statistical test to be performed depending on whether the sample follows a normal distribution or not. To determine if the values obtained previously followed a normal distribution, the Shapiro-Wilk test was used, since the sample size (N) was less than 50 in the comparisons made. If the significance level was more than 0,05 the sample was considered normal and, therefore, a T Student test was performed. However, if the significance level was less than 0,05, the sample was not considered normal and therefore the Mann-Whitney U-test was performed. A result was considered to be significant when the p-value was less than 0,05.

6 RESULTS

For more information, please contact with nagore.izal@estudiants.urv.cat

7 DISCUSSION

For more information, please contact with nagore.izal@estudiants.urv.cat

8 CONCLUSIONS

For more information, please contact with nagore.izal@estudiants.urv.cat

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