ROLE OF EPIGENETIC NON-CODING RNA MODULATION IN ALZHEIMER'S DISEASE MICE MODEL APP/PS1

TREBALL DE FI DE GRAU

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Grau de Nutrició Humana i Dietètica

Reus, 2023

AGRAÏMENTS

M'agradaria agrair al "Nutrition and Metabolic Health Research Group (NuMeH)" la calorosa acollida. Han fet que la realització d'aquest treball hagi estat plena d'aprenentatges, m'han obert les portes al món de la investigació i, sobretot, m'han acompanyat en els moments difícils.

En especial al Jaume, que des del primer moment m'ha donat totes les facilitats per participar del projecte que presentaré a continuació. I a l'Álex, que ha estat a peu de canó resolent els meus dubtes dia a dia.

NuMeH, quina sort que això no sigui un adéu, fins aviat.

Finalment, agrair també a totes aquelles persones que han fet que aquests 4 anys de carrera no s'hagin fet feixucs. Gràcies infinites a la família, amics i companys de classe; sense vosaltres hagués estat possible, però molt més complicat.

RESUM

Rol de la modulació epigenètica dels ARNs no-codificants en Malaltia d'Alzheimer en model de ratolins APP/PS1

Introducció: Els avenços en la ciència i en la tecnologia de les darreres dècades han comportat un canvi en la forma d'alimentació. Aquests canvis en la dieta han estat lligats a l'aparició de desequilibris metabòlics com la resistència a la insulina, donant lloc a un augment en la incidència Diabetis tipus 2. Al seu torn, la Diabetis ha estat relacionada amb la pèrdua de funció cognitiva i l'aparició d'Alzheimer. L'objectiu d'aquest estudi és identificar canvis epigenètic induïts per una dieta alta en greix sobre l'expressió de microARNs que predisposen l'aparició de la malaltia d'Alzheimer, com a possibles marcadors precoços de la malaltia.

Materials i mètodes: Es van obtenir mostres de sèrum i còrtex de ratolins *wild-type* i ratolins *APPswe/PS1dE9* (desenvolupen AD als 6-7 mesos de vida), dividits en 2 grups: un grup alimentat amb un 18% de l'energia provinent de greix i l'altre amb un 45% . A partir dels resultats de l'expressió dels miARNs d'estudi (146a-5p, 155-5p, 181c-5p, 19a-3p, 22-3p, 29c-3p i 320a-3p), es va procedir a fer un anàlisi estadístic per comparar els nivells d'expressió entre grups.

Resultats: Els miARNs 146a-5p, 22-3p, 29c-3p, 155-5p i 19a-3p van mostrar diferències significatives en l'expressió en almenys una de les comparacions realitzades.

Discussió: Els resultats d'aquest estudi proposen els miARNs 146a-5p i 19a-3p com a potencials biomarcadors precoços de la malaltia d'Alzheimer; així com un augment en la predisposició epigenètica de patir Alzheimer deguda a l'adhesió a una dieta HF. Tot i així, es necessita més investigació en aquest camp, per tal de poder aclarir algunes incògnites.

PARAULES CLAU

Malaltia d'Alzheimer, neuroinflamació, APPswe/PS1dE9, dieta alta en greix, resistència a la insulina, miRNA.

ABSTRACT

Role of epigenetic non-coding RNA modulation in Alzheimer 's disease mice model APP/PS1

Introduction: The advances in science and technology in the last decades have led a change in the diet pattern; which have been linked to the appearance of metabolic imbalances such as insulin resistance, leading an increase in the incidence of type 2 diabetes. Furthermore, diabetes has been linked to the loss of cognitive function and the onset of Alzheimer. The aim of this study is to identify epigenetic changes induced by a high fat diet on the expression of microRNAs which predispose to the onset of Alzheimer, as possible early markers of the disease.

Materials and methods: Serum and cortex samples were obtained from wild-type and APPswe/PS1dE9 (which develop Alzheimer at 6-7 months alive) mice, divided into two groups: one group fed with an 18% of energy coming from fat and the other one with a 45%. Based on the results of the expression of the miRNAs of study (146a-5p, 155-5p, 181c-5p, 19a-3p, 22-3p, 29c-3p and 320a-3p), a statistical analysis was done in order to compare miRNA expression levels between groups.

Results: The miRNAs 146a-5p, 22-3p, 29c-3p, 155-5p and 19a-3p showed significant differences in expression in at least one of the comparisons carried out.

Discussion: The results of this study propose miRNAs 156a-5p and 19a-3p as potential early biomarkers of Alzheimer's disease; as well as an increase in the epigenetic predisposition to suffer from Alzheimer due to the adherence to a high fat diet. Even tough, more research is needed in this field, in order to be able to clarify some unknowns.

KEYWORDS

Alzheimer's disease, neuroinflammation, APPswe/PS1dE9, high fat diet, insulin resistance, miRNA.

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1. INTRODUCCTION

Human nutrition has completely changed over the last 10.000 years, when the agricultural revolution started and especially since the industrial revolution 250 years ago [1], which have allowed the implementation of novel food and new culinary techniques. The term used to define the modern pattern of nourishment for western societies is called western diet (WD), which is based on ultra-processed food made from refined substances, rich in simple carbohydrates (mainly simple sugars), salt, fat (mainly saturated fatty acids (SFA)) and cholesterol. Moreover, this dietary pattern is poor in grain, fibre and monounsaturated and polyunsaturated fatty acids (MUFA, PUFA). Although there is no unified list of WD components, there is general agreement among those diets with 35-60% of fat with high concentration of SFA and a large amount of added sugar and cholesterol [2].

Furthermore the advances in science and technology have not only changed nourishment, but have also caused polluted environments, sedentariness and stress due to fast-paced life. All these changes in lifestyle constitute a fertile ground to increase the incidence of several diseases, such as obesity, diabetes, cardiovascular diseases, hypertension, osteoporosis and cancer. The term "civilization diseases" is derived from science and technology advances and is used to define all these pathologies.

The characteristics of WD and life style implanted since the progress in science and technology are a key point for the development of type 2 diabetes mellitus, led by high dietary intake of SFA, which is the major environmental factor associated with type 2 diabetes mellitus (T2DM) development [3].

1.1. Diabetes Mellitus

Diabetes mellitus (DM) is a worldwide chronic disease, which has become a serious global public health issue [4][5]. In 2017 there was a global prevalence of 425 million people with diabetes, which is expected to rise up to 629 million by 2045. This increase would be due to the global rise in the prevalence of obesity and unhealthy behaviours including poor diets and physical inactivity, led by the effects of the advance in technology and the WD [5]. There are two types of DM: type 1 DM (T1DM) and T2DM, this second one is the most prevalent accounting for more than the 80% of all the diabetes [4]. This increase in the prevalence is especially alarming since at the moment, there is no cure for T2DM and the disease can only be controlled by medical treatments [6].

While T1DM is an autoimmune disease caused by the response of the immune cells against β-cell which will induce their destruction and incapacity to produce insulin [7], T2DM is a disorder of carbohydrate metabolism characterized by hyperglycaemia [8], mainly caused by reduction of insulin effectiveness in lowering blood sugar levels. This requires the use of more insulin to metabolize blood sugar, known as insulin resistance [9][10]. In insulin resistance states, the β-cells of the pancreatic islets tend to respond by increasing insulin secretion to maintain normoglycaemia, a process named β cell compensation, which ends up with β-cell dysfunction and failure [11] which will result in hyperglycaemia and glucose intolerance [7].

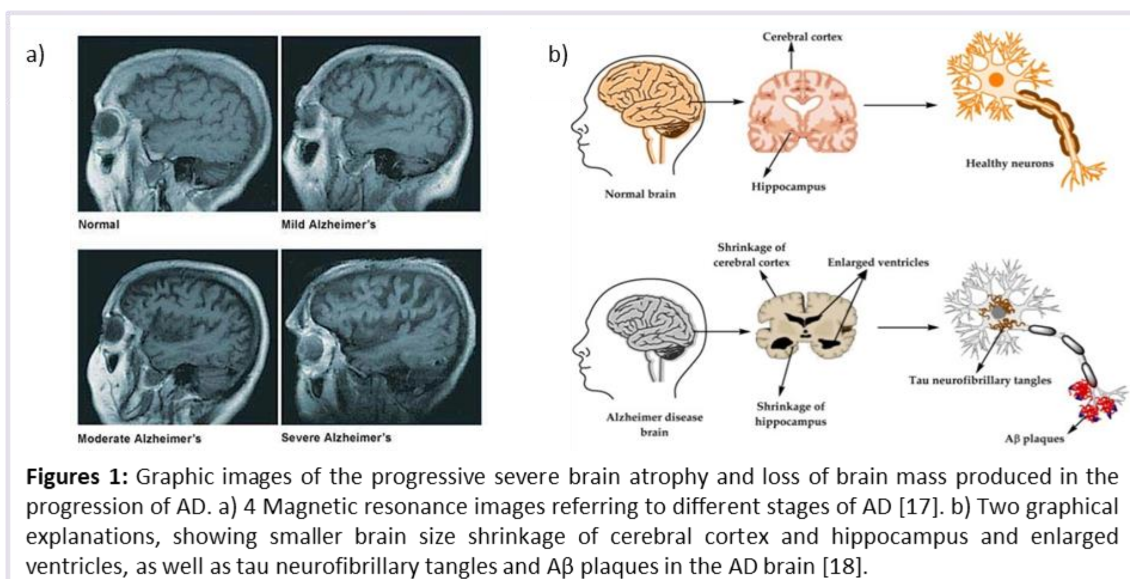
Furthermore, T2DM entails additional comorbidities such as hypertension, coronary heart disease, chronic kidney disease, chronic obstructive pulmonary disease, depression and it is also associated to cognitive decline, which could finally evolve to dementia [12][13].

1.2. Alzheimer disease

Alzheimer disease (AD) is a degenerative disease of the brain, characterized by the onset of dementia [14]. Dementia is a clinical syndrome characterized by a progressive decline in two or more cognitive domains, including memory, language, executive and visuospatial function, personality and behaviour; which causes a loss of the abilities to perform instrumental and/or basic activities of daily life [15].

1.2.1. Pathophysiology

AD appears mainly after the age of 60 and is pathologically marked by severe cortical atrophy [14] (figure 1). Even though the cause for most Alzheimer's cases still remains unknown, several proposed theories explain the pathophysiology of neurodegeneration [16].



Figures 1: Graphic images of the progressive severe brain atrophy and loss of brain mass produced in the progression of AD. a) 4 Magnetic resonance images referring to different stages of AD [17]. b) Two graphical explanations, showing smaller brain size shrinkage of cerebral cortex and hippocampus and enlarged ventricles, as well as tau neurofibrillary tangles and Aβ plaques in the AD brain [18].

Senile plaques: The most extended theory of AD's pathophysiology is the senile plaques (SPs) formation associated to neurodegeneration. The SPs are extracellular deposits of β-amyloid protein (Aβ) [18]. The β-amyloid plaques are the result of the cleavage of the amyloid precursor protein (APP) by the enzyme β-secretase [19]. According to this hypothesis, Aβ plaques are formed and get deposited in different regions of the brain. These plaques are recognised as a foreign material by the brain, initiating an inflammatory and immune response by activating the microglia and inducing the release of cytokines, which leads to neurodegeneration and cell death [20].

Neurofibrillary tangles: Neurofibrillary tangles (NFTs) are also a feature of AD. NFTs are composed with tau protein filaments which are abnormally hyperphosphorylated. The tau protein participates in maintaining the complex neuronal cell microarchitecture [21]. The filaments of this hyperphosphorylated protein can be twisted around each other in some stages, forming a paired helical filament (PHF) and getting accumulated in neuralperikaryal cytoplasm, axons and dendrites, which causes a loss of cytoskeletal microtubules and tubulin-associated proteins [22][23].

Synaptic loss: A synaptic damage in the neocortex is generally observed at the early stages of AD. There are different synaptic loss mechanisms which include defects in axonal transport, mitochondrial damage, oxidative stress and other processes that can contribute to the accumulation of Aβ plaques and tau tangles at the synaptic sites. These processes eventually lead to a loss of dendritic spines, pre-synaptic terminals and axonal dystrophy [24].

1.2.2. Stages of Alzheimer's disease

The clinical phases of AD can be classified into 3 stages:

- The first one is the **pre-clinical or the pre-symptomatic stage**, which can last for several years. This stage is characterized by mild memory loss and early pathological changes in cortex and hippocampus, with no functional impairment in the daily activities and absence of clinical signs and symptoms of AD [25][26].
- The next stage is the **mild or early stage** of AD. In this stage the symptoms start appearing; such as complications in daily life, loss of concentration and memory, disorientation, a change in the mood and a development of depression [27].

- The third stage is the **moderate** AD, when the disease is spread to cerebral cortex areas. In this stage there is an increased loss of memory and trouble recognizing family and friends, a loss of impulse control and difficulty in reading, writing and speaking [28]. In the severe or late-stage AD, takes place the spread of the disease to the entire cortex area with a severe accumulation of SPs and NFTs. In this stage the patients cannot recognize their family and may develop difficulties in swallowing and urination [25][29].

1.2.3. Epidemiology

AD is the most common dementia in developed countries, being one of the main causes of death, disability and dependency in the elder population. In 2020, there were an estimated 50 million Alzheimer's patients worldwide and this is expected to increase to 152 million by 2050. The burden of the disease affects individuals, their families and the global economy [18].

This pathology is considered a multifactorial disease with several risk factors such as increasing age, genetic factors, head injuries, vascular diseases, infections and environmental factors; which play an important role in AD [18].

Currently, although patients receive symptomatic treatment with drugs in order to delay its progression, there are no effective therapies to cure AD [15]. Even though, different preventive measures can help reduce risk factors, such as following a proper diet and being physically and mentally active [30][31]. Preventive measures introduced before the onset of dementia symptoms, by the age of 50, are the ones that have shown better results, which results in the increase of chances to stay healthy for a longer period of time [32].

1.2.4. Diet and Alzheimer's disease

Scientific evidence has linked different dietary patterns with the onset of AD. A Mediterranean diet rich in antioxidants, fibre and omega 3 polyunsaturated fatty acids has shown a protective effect on the neurodegenerative process of Alzheimer [33], whereas those dietary habits that lead to the appearance of cardiovascular and metabolic diseases have been shown to significantly increase the risk of dementia [30][34].

The excessive intake of saturated fatty acids (SFA) can aggravate cognitive impairment. On the other hand, the consumption of large amounts of food rich in monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), especially from the omega 3 family, significantly decrease the risk of dementia [33][35]. The properties of fatty acids depend on the length of their chain and the number of double bonds. Additionally, PUFAs are one of the main components of neurons, hence their importance for the proper functioning of cognition. Otherwise, MUFAs and PUFAs act as antioxidants, because of their double bonds [36]. Some examples of food rich in unsaturated fatty acids are olive oil, nuts, avocado, etc; whereas the saturated fatty acids are commonly found in foods such as pastries, coconut oil and rich in animal fat food.

1.3. Type 2 Diabetes Mellitus and Alzheimer disease

Two of the epidemics of our time, T2DM and AD, seem to be related. Actually, patients with T2DM have around 60% higher risk for the development of dementia compared with those without diabetes [37].

The cognitive decline originated by T2DM arises from the importance of insulin in the brain functionality, where influences cerebral bioenergetics, enhances synaptic viability and dendritic spine formation, as well as increases turnover of some neurotransmitters. Moreover, insulin also has a role in proteostasis, influencing clearance of the β-amyloid peptide and phosphorylation of tau, features of AD. These multiple pathways could contribute to neurodegeneration when an insulin dysregulation stage is established [38][101].

Some animal models of neurodegeneration such as APPswe/PS1dE9 double transgenic mice helped for a better understanding of these associations between metabolic imbalances and neurodegeneration. In fact, the exposure of mice to high fat diet obesity allowed finding molecular mechanisms shared by the two disorders. Furthermore, evidence has linked obesity and AD biomarkers with inflammation, adipokine dyshomeostasis, oxidative stress and mitochondrial dysfunction; all mechanisms leading to neurodegeneration [102].

1.4. Biomarkers

Regarding the increasing rise of AD and also of T2DM, which seems to increase the proneness to develop AD, and the heavy social impact of AD for the patients, families and the global economy; it is necessary to improve its diagnosis and treatment.

Nowadays the diagnosis of AD requires the presence of specific clinical phenotype and biomarker evidence [39]. On one hand, the main clinical phenotype is the amnestic one, which targets episodic memory, but also there are other rare phenotypes [40]. On the other hand, the biomarkers required to diagnose AD are Aβ peptides and phosphor-tau, which are measured in the brain by imaging methods or in the cerebrospinal fluid by ELISA assays [39][41].

When trying to find novel biomarkers to assess the progress and prognosis of AD disease, epigenetics arise as a promising field to be explored. Epigenetics is the study of the modifications caused by behaviour and the environment, such as diet, which directly affect the expression of genes, without producing changes in the DNA sequence [42]. Alterations in the epigenetics are involved in the pathogenesis of several diseases, including AD [43].

Research into epigenetic alterations causing AD has focused on 3 pathways: methylation and hydrometylation of DNA, post-translational modifications of histones and regulation of non-coding RNA (ncRNA) [43].

1.4.1. non-coding RNA

ncRNA are RNAs which are not translated into proteins but they are crucial in the regulation of numerous cellular functions, by modifying the activity of DNA, RNA and proteins. This results in influencing the genetic expression, the translation of messenger RNA (mRNA) and the formation of protein complexes [44][45]. Two types of ncRNAs can be distinguished: housekeeping and regulators. Housekeeping ncRNA are transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA). Regulatory ncRNA are important for the regulation of gene expression and can be classified according to their length into short-chain ncRNAs (small interfering RNAs, microRNA (miRNAs) and piwi-interacting RNAs) and long-chain ncRNAs (lncRNAs) [46].

1.4.2. microRNA

miRNAs are single chain RNAs of 19 to 29 nucleotides in length with an important role in post-transcriptional gene silencing. They destabilize mRNAs by an imperfectly binding to the corresponding sequences, which are usually found in the 3'-untranslated regions of the mRNAs in question [47]. The result of the action of miRNAs is the translational repression or degradation of the target mRNA [48].

More than 2000 different miRNAs are known to regulate gene expression in humans, each of which can regulate the expression of hundreds of different genes, and induce various histones modifications [59].

The miRNA biogenesis (figure 2) requires two steps. The first one takes place in the nucleus, where the DNA is transcribed to primary miRNA (pri-miRNA) [52], leaded mostly for the RNA polymerase II [51]. This pri-miRNA is long and has a terminal loop in 5' and a tail of poly-adenines (poly-A) in 3' site. Afterwards, the Microprocessor complex, formed by nuclear RNase III DROSHA and a cofactor known as PASHA, crops the stem-loop to release a small structure of about 65 nucleotides, which are called pre-miRNA. The protein exportin 5 (EXP5) forms a transport complex with guanosine triphosphate (GTP) binding the ras-related nuclear protein (RAN·GTP) and a pre-miRNA. This complex transports the pre-miRNA to cytoplasm, were the second step takes place. The pre-miRNA is processed to mature miRNA by the ribonuclease DICER [51].

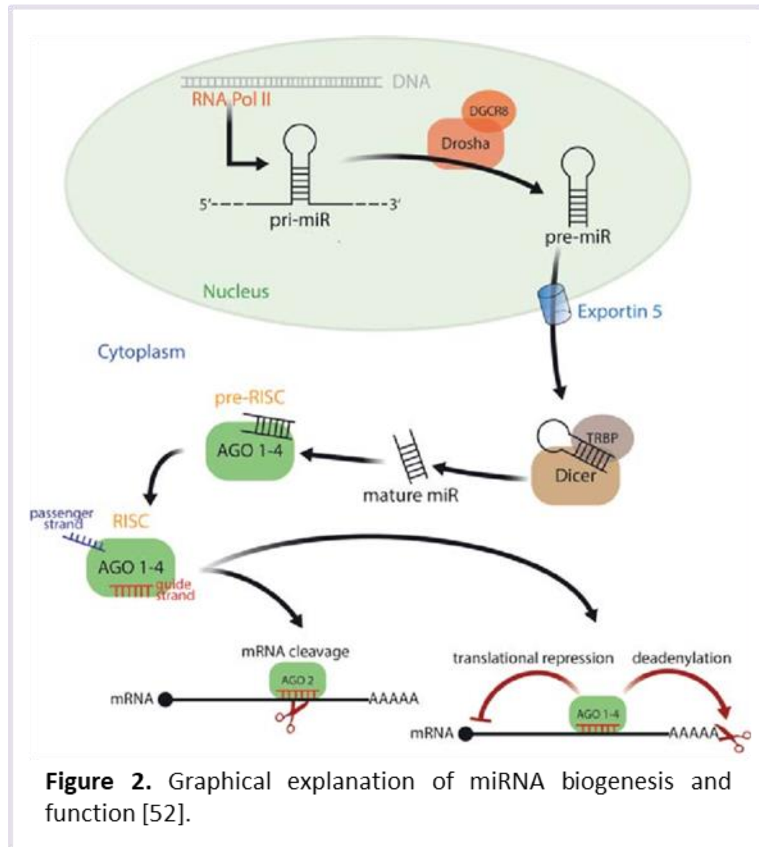


Figure 2. Graphical explanation of miRNA biogenesis and function [52].

DICER also initiates the formation of ARN-induced silencing complex (RISC), which is responsible of the gene silencing caused by miRNAs expression. One of the miRNA strands is integrated to the RISC complex, named guide strand, and the other strand, called passenger strand, is degraded by RISC. Finally, the guide strand binds to the mRNA of the gene to silence it and can repress gene expression by 4 ways: protein degradation during translation, inhibition of translation elongation, producing a premature termination of translation or inhibition of the translation [50][51].

Circulating miRNAs can be detected in the peripheral circulation (serum, plasma, exosomes, blood) and also in the cerebrospinal fluid [53]. On the other hand, non-circulating miRNAs are found in brain tissues (hippocampus, cerebellum and temporal, frontal and parietal cortex). The miRNAs that can be found both in the peripheral circulation and in the central nervous system are the most interesting, due to their potential as significant biomarkers [54] and their non-invasive collection.

Due to their involvement in the regulation of many pathways, miRNAs have been postulated as good markers for the diagnosis, prognosis and to develop therapies for different pathologies [49].

In summary, T2DM and AD are both high prevalence diseases and cause a huge impact on patients' lives, relatives and global economy. On one hand, both diseases are linked to diet. The recent changes in the food industry and consequently in the diet pattern, have coincided with an increase of both pathologies incidence. On the other hand, the diagnosis of AD is detected in late stages of the pathology, whereas an earlier diagnosis could slow down the onset of serious complications. Taking everything together, genetic markers, such as miRNAs, seem to be interesting in the diagnosis of AD, which can be detected earlier and in a less invasive way. Furthermore, epigenetics can help to establish relationship between the diet pattern and genetic expression.

1.5. Aim and hypothesis of the study

The aim of the current study is to identify epigenetic changes induced by a diet pattern associated to insulin resistance, high fat diet, on the expression of microRNAs which predispose the onset of AD.

The hypothesis of this study is that a HF diet pattern causes epigenetic changes that predispose to develop AD; which means that a diet can be part of prevention and miRNAs can be used as early biomarkers.

2. MATERIALS AND METHODS

2.1. Mice, diet and sampling

The present study was performed using mice (n= 59) with different genotypes. The wild-type C57BL/6J mice (WT) (n=31), which is the most commonly used for research; and the transgenic APPswe/PS1De9 mice (APP) (n=28), which has a double mutation associated to the development of AD. This double mutation lead to develop spontaneously β-amyloid protein deposits in the brain by 6 to 7 months of age [55][56].

The mice were separated into two study groups, one group fed with a control diet (CT) and the other one with a high-fat diet (HF). The HF diet was purchased from Research Diets, Inc (Product D12492) [57], a rodent diet with the 60% of the total energy provided from fat, mainly lard fat. On the other side, the CT diet only provided the 18% of the energy from fat.

	CT	HF
	Kcal %	**Kcal %**
Protein	24.0	16.4
Carbohydrate	58.0	38.6
Fat	18.0	45.0
Ingredients		**g**
Casein, 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
Vitamin Mix V10001		40

Table 1. Macronutrient distribution for the two different diets of study (CT and HF), as well as the ingredients of the HF diet.

All the mice were fed *ad libitum* with these diets (**table 1**) from weaning to 16 months of life, when they were sacrificed.

The animals were kept under controlled temperature, humidity and light conditions with food and water ad libitum. Mice were treated in accordance with the European Community Council Directive 86/609/EEC and the procedures established by the *Department d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya*. Every effort was made to minimize animal suffering and reduce the number of animals used.

Immediately the samples of serum and cortex were obtained, frozen and stored at -80ºC until further processing. The aim of the samples processing was to quantify the miRNA expression according to groups of study. To make sure the results were not altered by external factors, negative and positive controls were also analysed. A miRNA from *Caenorhabditis elegans* was used as a positive control [58] and a miRNA from *Arabidopsis thaliana* was used as a negative control [59][60].

2.2. RNA extraction

Total RNA extractions of serum and cortex samples were performed to analyse the selected miRNAs expression, using the mirVANA PARIS Kit as indicated by the provider. The kit allows the disruption of the original tissue employing a buffer (Cell Disruption Buffer) and obtaining a lysate. The further use of a denaturing solution and phenol chloroform acid facilitates the RNA purification and the elimination of DNA through a separation into two phases; an inorganic phase, with the RNA, and an organic phase with the remaining. Afterwards, the inorganic phase was collected and passed through an affinity column (filter cartridge) which hybridized the RNA molecules. The application of a few washes with ethanol solutions allowed the purification of the RNA for each sample. In the last step, a low ionic strength solution (RNAse free water) was applied to separate the RNA fraction from the column and elute it. Finally, the total RNA concentration of each sample and their quality was assessed by Nanodrop [61] and the samples were stored in a freezer at -80ºC until further processing.

At the start of the RNA extractions, the exogenous miRNA (spike in) from *Caenorhabditis 2 elegans* was added to the samples considering a proportion of 3:200 for all the samples.

The protocols were executed identically regardless the nature of the sample, except of the following steps. The cortex samples were weighted and homogenized using the IKA™ ULTRA-TURRAX™ T 18 DIGITAL DISPERSER [62] before the processing. Furthermore, the Cell Disruption Buffer was used only for the cortex samples; since it is only used for solid samples such as the cortex ones.

2.3. cDNA synthesis

For the cDNA synthesis the TaqMan® Advanced miRNA cDNA Synthesis Kit was used. The aim of the cDNA synthesis is to use the total RNA isolated in the previous step for each sample to synthesise a complementary DNA for all the miRNAs [63].

To achieve the purpose of the protocol, a Poly(A) tailing reaction uses poly(A) polymerase to add a 3'-adenosine tail to the miRNAs. The miRNAs with poly(A) tail underwent an adaptor ligation at the 5' end, where the adaptor acts as the forward-primer binding site for the following miR-Amp reaction. Then, an universal reverse transcription (RT) primer binds to the 3' poly(A) tail and the miRNA is reverse transcribed. Finally, in the miR-Amp reaction, universal and forward reverse primers increase the number of cDNA molecules and ensures the concentration to be within the exact ranges for the qPCR [64].

The whole protocol was performed with 10ng of RNA. Then, the steps of the Poly(A) Tailing Reaction, the Adaptor ligation Reaction, the Reverse Transcription and the miR-Amp Reaction were done as described in the the TaqMan® Advanced miRNA cDNA Synthesis Kit. Each step requires mixing the RNA samples with a reaction mix and applying incubation with a thermal cycler [65]. All the compounds and the thermal cycles employed within the protocol are explained in annex 2.

2.4. Quantitative real-time PCR

The next procedure consists of performing a polymerase chain reaction (PCR), which allows genetic changes to be observed. This procedure has the function of amplifying a small amount of genetic material [66]; in this case, the miRNAs of study. Quantitative PCR (qPCR) allows not only to determine whether the sequence of genes of interest (GOI) is expressed, but also to quantify GOIs' expression [67].

Before starting the qPCR, the samples were diluted with nuclease-free water to 1:10 dilution. Then, 2,5µL of the dilution were put into each well of the MicroAmpTM Optical 384-Well Reaction Plate [68](figure 3) with 5µL of Mastermix, 0,5µL of primer and 2µL of water as described in the TaqMan® Fast Advanced Master Mix protocol [69]. The primers used in the present protocol are designed specifically for TaqMan Gene Expression Assays [70].

The Mastermix consists of TaqMan probes, Taq polymerase, dNTPs and buffer reagents. TaqMan probes are fluorescents chemical markers with oligonucleotide that bind to a specific nucleotide sequence [70].

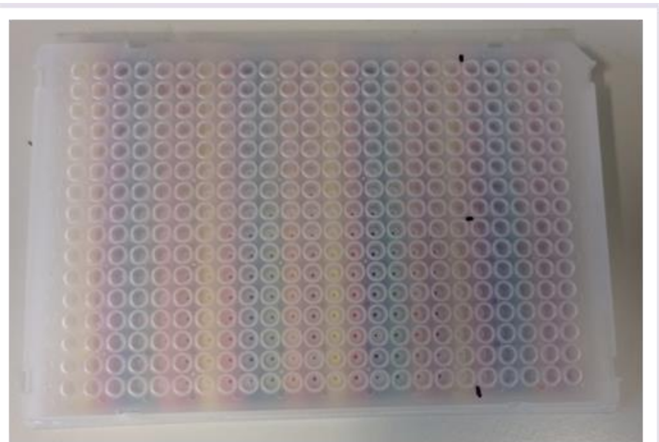


Figure 3. qPCR plaque. Each line was for a different sample, and in the columns were putted the specific primers for each miRNA of study.

As with standard PCR, qPCR is performed using a thermal cycler, which can rapidly heat and cool samples to allow replication of GOIs. As it can be seen in the annex 3, the thermal cycler performs 2 steps (figure 4). The first one allows the enzyme activation, which is one cycle of 20 seconds and the second step is formed by 40 cycles. Each cycle starts with a denaturing step. Later, the primer binds the complementary strand of target cDNA and the probe interacts with the target nucleotide sequence [67]. The probe is formed by a nucleotide sequence, a fluorophore which is attached to one end of the probe (reporter) and has the ability to absorb energy from light, and a non-fluorescent quencher is attached to the other end, which has the ability to cause the reporter not to be enlightened [71]. Finally, the elongation is performed. The amplification of cDNA as the PCR cycles progress enables the fluorophore to separate from the quencher, which subsequently results in an increase in fluorescence, the intensity of which can be analysed afterwards.

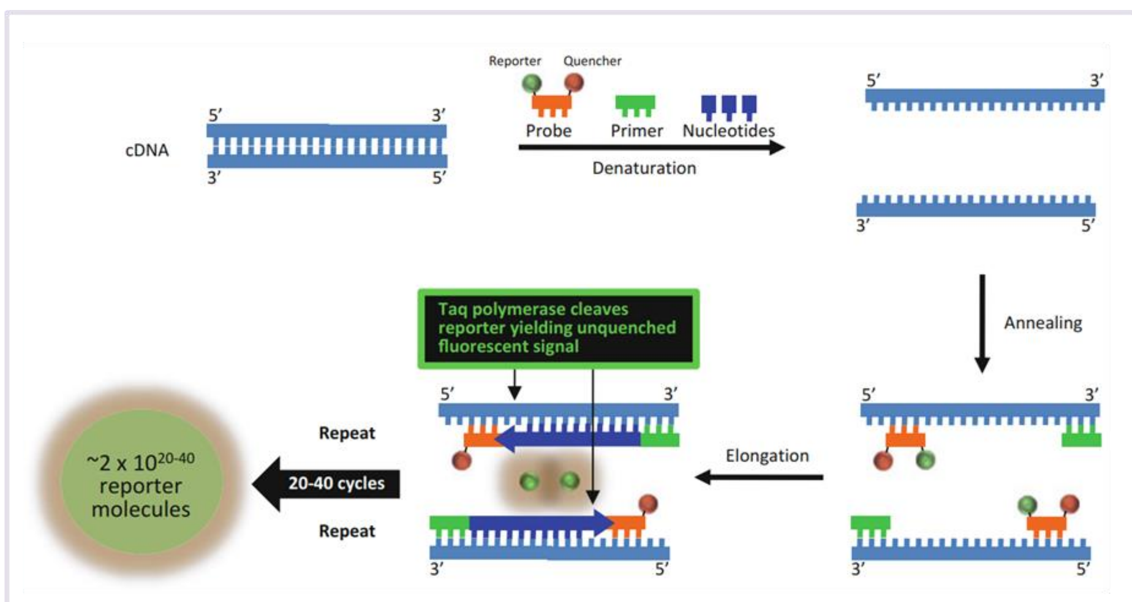


Figure 4: Schematic diagram of the qPCR procedure [72].

In order to select those miRNAs which were more interesting for our study, a bibliographic research allowed to identify those miRNAs related to AD and T2DM. The list of miRNAs selected was 146a-5p, 155-5p, 181c-5p, 19a-3p, 22-3p, 29c-3p and 320a-3p; explained in table 2.

miRNA	Expression in Alzheimer	Expression in T2DM
hsa-miR-146a-6p	Upregulated in neurological disorders associated with progressive age-related inflammatory neurodegeneration [73][74].	An important insulin regulator [75], which supress adipogenesis. The dysfunction of adipose tissue will lead to a series of metabolic syndromes, such as insulin resistance and T2DM [76].
hsa-miR-155-5p	Increases Aβ aggregation and aggravates AD. Its inhibition ameliorates cognitive impairment, improves neuron regeneration and attenuates Aβ deposition [77].	Controls adipose tissue accumulation by affecting energy metabolism. An increase of this miRNA reduces adipocyte differentiation and causes hyperinsulinemia and insulin resistance [78].
hsa-miR-181c-5p	Regulates the activity of tau protein, which is involved in the pathogenesis of AD [79]. Down regulated in AD.	Contributes to glycogen synthesis and mediation of insulin resistance [80]
hsa-miR-19a-3p	Contributes in neuroinflammation causing neurodegeneration [81]. Downregulated in AD patients [82]	Downregulated in diabetes. Increases insulin secretion and pancreatic β cells apoptosis [83].
hsa-miR-22-3p	Downregulated in AD [84], which leads the activation of proinflammatory cytokines [85].	Upregulated in metabolic disorders such as diabetes [86], increasing the expression of enzymes of the gluconeogenic pathway [87].
hsa-miR-29-3p	Downregulated in AD. Increases the expression of β-site amyloid precursor protein cleaving enzyme 1 (BACE1) [88].	Upregulated in diabetes. Regulates the expression of genes participating in glucose metabolism such as GLUT4 and HK2 [89].

hsa-miR-320a-3p	Downregulated in AD. The overexpression of this miRNA may attenuate Aβ-induced neurotoxicity and improve the cognitive function [90].	Overexpressed in diabetes. Increase ROS level, inhibits proliferation and induces apoptosis of β cells [91].

Table 4. Relationship between miRNAs 146a-5p, 155-5p, 181c-5p, 19a-3p, 22-3p, 29c-3p and 320a-3p with AD and/or T2DM.

The expression of these seven miRNAs was reflected on the graphs obtained from the qPCR (figure 5), which show the amplification curves of each miRNA.

2.5. Statistical analysis

Based on the graphs obtained in the qPCR, it is necessary to establish a fluorescence threshold. The point where the amplification curve of the study miRNAs meets the threshold is called cycle threshold (Ct) (figure 5) and allows obtaining the measurements to know the relative expression levels of each miRNA [92]. Additionally, the expression of the spike in, which has to be proportional to each sample, and the miRNA from *Arabidopsis thaliana*, which should not be present within the samples, were also considered.

From the Cts of each miRNA, normalization was done in relation to an exogenous control, in this case the spike in added in the RNA extraction which allows having a known miRNA Ct standardized for all the samples. This normalization is called ΔCt. The ΔCt of a specific sample was calculated by the subtraction of spike in Ct to each miRNA of interest Cts, both from the same sample [94].

ΔCt = Ct (miRNA of interest) – Ct (Spike in)

In order to continue with the statistical analysis, those extreme values that differ from the majority of values in the dataset that belong to the same group must be detected and excluded. The z-sore test was the chosen method, which consists of calculating a standardized value of each sample in relation to the mean values of the miRNA values [93].

Z-score = (sample dct value – mean of all miRNA samples)/standard deviation of miRNA values

Then, those values higher than 2,5 or lower than -2,5 were excluded and considered outliers.

The ΔCts were used to make comparisons between groups which included: two comparisons between diets for the same genotype (APPHF vs APPCT and WTHF vs WTCT) and another comparison to show differences between genotypes (APPCT vs WTCT). With the aim to compare groups, the difference between the ΔCt of one sample and the average ΔCt of the normalization group (APPCT or WTCT according to each comparison) was calculated [94].

ΔΔCt (sample x) = ΔCt (sample x) – average ΔCt (group normalizing the sample x)

The last calculation was the fold change, which was obtained raising 2 to the negative ΔΔCts. Afterwards the statistical test can be applied to evaluate the expression differences between groups [94].

Fold change = $2^{-\Delta\Delta Ct}$

Then, the logarithm fold change was calculated in order to identify correctly the biological fold change relevance. Since, in certain cases, a miRNA with a significant p-value can have a rather small fold change while a non-significant miRNA can have a rather large fold change [95]. The log fold change can be found in annex 5.

The following step was to do a normality test, which allows knowing whether the measures follow a normal distribution or not. For all the samples the test executed was the Shapiro-wilk [96]. P value higher than 0,05 in the normality test indicates that the values follow a normal distribution [97].

The remaining step to complete the statistical analysis is the application of statistical tests for the three comparisons (APPHF vs APPCT, WTHF vs WTCT and APPCT vs WTCT), separating the serum from the cortex samples. In order to choose the statistical test it is important to detect if the variables are paired or independent. Since the samples belonged to different mice, all the variables were regarded to be independent. Another important aspect to consider is the distribution of the measures. According to the results from the normality test those comparisons with normal distribution measures were done with the parametric T-test, whereas those comparisons without normal distribution measures were done with the non-parametric U de Mann Whitney [97].

2.6. Functional enrichment analysis

Once the miRNA expression was quantified and statistically analysed, the research of findings started, which could help to understand the physiological pathways altered by our miRNA of study expression or repression.

The first step consisted on identify those miRNAs with significant differences between groups for each comparison, in order to find those significant miRNAs in more than one comparison. Venn diagrams were drawn in http://www.interactivenn.net .

Afterwards, the minimum network with the strongest evidenced target-genes of miRNAs linked to the neurodegeneration promoted by the exposure to a HF diet in AD mice model APP/PS1 signatures were identified through the validated miRTarBase [98] database, incorporated in miRNet platform [99].

In order to clarify the potential function annotation and pathway enrichment associated with the significate miRNAs as a whole, Gene Ontology (GO) analyses, including biological process (BP), molecular function (MF), and cellular component (CC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, were performed to figure out the functional roles of these miRNAs in WebGestalt platform [100].

Finally, a literature search using the MEDLINE-PubMed database to extract gene functions related to MCI or AD, allowed correlating the results from these studies with the previous evidence.

3. RESULTS

The following results are aimed to clear up the hypothesis of the study, in order to understand the effect of the HF diet in the expression of selected miRNAs related to progression of neurodegeneration. Furthermore those miRNAs expressed in both cortex and serum would be particularly interesting as biomarkers of AD.

3.1. Effect of a HF diet on miRNA expression in APP/PS1 mice

Meaningful statistical change was observed in the miRNA 146a-5p and the miRNA 19a-3p.

3.2. Effect of HF diet on miRNA expression in WT mice

Meaningful statistical change was observed in the miR-19a-3p, the miR-22-3p and the miR-29c-30 in the APPHF vs APPCT comparison.

3.3. Differences in miRNA expression between WT and APP/PS1 genotypes

Meaningful statistical change was observed in the miR-155-5p and the miR-19a-3p in the WTCT vs APPCT comparison.

3.4. Functional enrichment analysis

The **Venn diagram** showed that miRNA 146a-5p was significantly up-regulated when the APP mice was exposed to a HF diet; the miRNAs 22-3p and 29c-3p were significantly up-regulated when the WT mice was exposed to a HF diet; the miRNA 155-5p was significantly up-regulated when comparing APP and WT genotypes; and finally, the miRNA 19a-3p presented significant differences in expression in the three comparisons. Furthermore, the miRNA 19a-3p was significantly overexpressed in both serum and cortex samples.

The validated target genes of all the five significant miRNAs were obtained from **miRTarBase**. All those target genes were introduced into **WebGestalt**, obtaining the functional enrichment.

The strongest target genes were obtained from **miRnet** platform.

4. DISCUSSION

AD is a neurodegenerative disease, which is characterized by the progressive decline of cognition. The loss of memory, language abilities, executive and visuospatial function, personality and behaviour cause radical changes in the lives of the people affected by this pathology [14][15]. Nowadays, it is an incurable disease and the treatment aims to slow down the degeneration of the brain, in order to delay the onset of more cognition decline [15]. In this way, the earlier the detection of the pathology, the more delay of the onset of serious complications [32]. However, the current diagnosis techniques can only detect AD in those patients in mild or moderate stages, when the symptoms have already appeared [39][41].

The medical research in AD is focused on finding new diagnostic techniques which allow the detection of the disease before the advanced appearance of symptoms, as well as the study of prevention measures that avoid the appearance of symptoms. In this direction, AD risk factors seem to be an interesting field [30][31]. Their study can allow the finding of new preventive strategies and the discovery of new AD markers, which can be detected in early stages of the pathology.

The present study has focused on HF diet as a modifying risk factor which has shown to have an important role in AD pathogenesis; specifically on its role promoting epigenetic changes in miRNA expression, which could be used as early AD markers [49]. In order to study whether a HF diet promotes a miRNA pattern which is related to AD pathogenesis, rodent model individuals were used with different genetic background and fed with different diets. Consequently, there were performed three comparisons. One of them allowed comparing differences in miRNAs expression pattern between wild type mice (WT) and mice with genetic predisposition to develop AD (APP); both fed with a control diet. The other two comparisons allowed showing the epigenetic modifications produced by the HF diet in mice with the same genotype (in APPHF vs APPCT and WTHF vs WTCT comparisons) and comparing those miRNA expression patterns with the ones in the control groups.

The statistical analysis showed that miRNA 146a-5p was significantly up-regulated when the APP mice were exposed to a HF diet; the miRNAs 22-3p and 29c-3p were significantly up-regulated when the WT mice were exposed to a HF diet; the miRNA 155-5p was significantly up-regulated when comparing APP and WT genotypes; and finally, the miRNA 19a-3p presented significant differences in expression in the three comparisons.

Taking into account these promising results, the present study could be a start point for other studies to shed more light into the field and describe whether the miRNAs play a pivotal role in the development of AD. Some of the factors that can be considered to obtain more accurate results could be increasing the sample size, obtain more parameters such as weight, blood glucose levels, insulin resistance and cognitive function indices.

Finally, the results of this study suggest miR-146a-5p and miR-19a-3p as potential biomarkers for different reasons. On the one hand, miR-146a-5p is largely linked to AD physiopathology via targeting CHF and TIGAR genes. Moreover, the results of the current study also linked a HF with an increase of this miRNA expression. On the other hand, there is no strong evidence explaining the paper of miR-19a-3p in the physiopathology of AD, such as its regulation (upper or downregulation) or the target genes involved on AD development. It can be concluded that miRNA study is a promising field to find serum biomarkers of AD; moreover, the understanding of the epigenetic changes in miRNA produced by diet can be useful in AD prevention. In order to get those results there is a necessity of further research, which includes a larger number of samples as well as parameters about weight, blood glucose levels and cognitive function. Furthermore, taking into account the large number of genes targeted by a unique miRNA and the wide role in different pathologies; it is necessary further research focused on finding a group of significant miRNAs, the expression of which predisposes to AD development. Integrating the data obtained from platforms and databases such as miRnet, miRTarBase, WebGestalt and Geneontology can be useful to understand the behaviour of miRNAs.

The results from further studies could allow linking a dietary pattern such as HF diet, already related to metabolic diseases such as diabetes, since it promotes insulin resistance, with epigenetic modulations in miRNA expression which prones to develop AD. These findings would entail a huge advance in AD's prevention and treatment, due to the chances of preventing a disease with such a high impact on society by solely modifying the diet. Furthermore, the knowledge of specific miRNAs of AD could allow an early and non-invasive detection, which would enable an earlier start of the treatment and delay the onset of serious complications.

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