THE EFFECTS OF DIFFERENT DIETARY INTERVENTIONS AND AGEING ON DNA METHYLATION LEVELS OF SIRT1 IN THE BRAIN OF MAMMARY TUMOUR MOUSE MODEL

by
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ABSTRACT

THE EFFECTS OF DIFFERENT DIETARY INTERVENTIONS AND AGEING ON DNA METHYLATION LEVELS OF SIRT1 IN THE BRAIN OF MAMMARY TUMOUR MOUSE MODEL

Sirtuin 1 (SIRT1) is a NAD^+-dependent deacetylase that maintains cell homeostasis by playing an important role in cellular processes, such as gene transcription, stress reaction and apoptosis. Dietary interventions such as calorie restriction (CR) modulates SIRT1 activity and that leads to increment in healthspan and lifespan in many organisms. The preservation of brain functions that decline with ageing is positively impacted by SIRT1. The aim of this study was understanding the biological link between DNA methylation of *Sirt1* and effects of different types of dietary interventions and a causal role of ageing in mice brain. To investigate *Sirt1* gene methylation due to ageing and CR, 10 weeks old female C57BL/6 mice were fed ad libitum (AL), with chronic calorie restriction (CCR) (15% CR) or intermittent calorie restriction (ICR) (three weeks of AL followed by one week 60% CR in a cyclic manner). Pyrosequencing was performed to examine the methylation levels of *Sirt1* from brain homogenates and understand the underlying mechanisms of CCR and ICR in relation to ageing at the genomic level. Western-blot was used to determine protein expression levels whereas RT-PCR was used to determine *Sirt1* mRNA levels. We could not find any significant difference in expression and DNA methylation levels of *Sirt1* with ageing. Also, the diet-associated mRNA and protein expression levels and DNA methylation changes of *Sirt1* were not obtained. A significant negative correlation was observed between DNA methylation levels and mRNA/protein expression of *Sirt1*. In addition, a statistically significant positive correlation was found between protein expression and mRNA expression. These findings indicate that decrease of SIRT1 by ageing might be regulated at the proteomic levels or with other epigenetic mechanisms. DNA methylation has a regulative effect on *Sirt1* expression regardless of diet and age.

ÖZET

FARKLI ŞEKİLLERDEKİ DİYET UYGULAMALARININ MEME TÜMÖRÜ FARE MODELİNİN BEYNİNDEKİ SIRT1 DNA METİLASYONU ÜZERİNDEKİ ETKİSİ

Sirtuin 1 (SIRT1), gen anlatımı, yaşam süresinin belirlenmesi, stres yanıtı ve apoptoz gibi belirli hücresel süreçlerde hücresel homeostazı koruyan bir NAD^+ bağımlı deasetilazdır. Kalori kısıtlaması (KK) gibi diyet müdahaleleri SIRT1 aktivitesini modüle eder ve memelilerde yaş ve metabolizma hızında artışa neden olur. SIRT1, yaşlanma sırasında kötüleşen nörolojik süreçlerin korunmasında olumlu etkiye sahiptir. Bu çalışmanın amacı farklı şekillerde uygulanan kalori kısıtlamasının ve yaşlanmanın fare beynindeki *Sirt1* DNA metilasyonu üzerindeki etkisini araştırmaktır. Yaşlanma ve diyet müdahalelerine bağlı *Sirt1* gen metilasyonunu araştırmak için 10 haftalık dişi C57BL/6 fareler, ad libitum, sürekli kalori kısıtlaması(SKK) (AL grubuna kıyasla yüzde 15 kalori kısıtlaması) veya aralıklı kalori kısıtlaması (AKK) (döngüsel bir şekilde üç haftalık AL ve AL grubuna kıyasla bir hafta yüzde 60 kalori kısıtlaması) ile beslendi. Pyrosequencing tekniği farelerin beyinlerinden izole edilen DNA'lardaki *Sirt1* metilasyon seviyelerini ölçmek için kullanıldı. *Sirt1* mRNA ekspresyonu, RT-PCR ile ölçülürken, protein seviyeleri western-blot ile karşılaştırıldı. *Sirt1* gen ve protein ekspresyonunda ve DNA metilasyon seviyesinde yaşlanmaya bağlı olarak istatiksel olarak anlamlı bir fark gözlemlenmedi. Farklı kalori kısıtlaması uygulanan gruplar arasındaki *Sirt1* ekspresyonu ve DNA metilasyon seviyelerinde değişiklik tespit edilmedi. DNA metilasyon seviyesi ile *Sirt1* mRNA ekspresyonu arasında istatiksel olarak anlamlı negatif bir korelasyon bulundu. Bu sonuçlar, yaşlanma ile hücrelerde SIRT1 miktarındaki azalmanın proteomik seviyedeki düzenlemelere veya diğer epigenetik mekanizmalara bağlı olabileceğini düşündürmektedir. DNA metilasyonu, diyet ve yaştan bağımsız olarak *Sirt1* ekspresyonu üzerinde düzenleyici bir etkiye sahiptir.

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LIST OF SYMBOLS/ABBREVIATIONS

°C	Degree centigrade
µl	Microliter
ACTB	Actin beta
AD	Alzheimer's Disease
ANOVA	Analysis of variance
BRCA	Breast cancer gene
BSA	Bovine serum albumin
CCR	Chronic calorie restriction
cDNA	Complementary DNA
CR	Calorie restriction
Ct	Cycle threshold
CVD	Cardiovascular disease
dATP	Deoxyadenosine triphosphate
DE	Differentially expressed
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
ER	Estrogen receptor
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor
ER	Estrogen receptor
FOXO	Forkhead box O
g	Gravitational force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
h	Hour
ICR	Intermittent calorie restriction
ICR-R	Intermittent calorie restriction-restriction
ICR-RF	Intermittent calorie restriction-refeed
IGF-1	Insulin-like growth factor 1
mRNA	Messenger RNA

min	Minutes
mM	Millimolar
MMTV-TGFα	Mouse mammary tumor virus-transforming growth factor-alpha
NAD	Nicotinamide adenine dinucleotide
ng	Nanogram
PBS	Phosphate-buffered saline
PD	Parkinson’s disease
PTHM	Post-translational histone modification
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM	Standart error mean
SGZ	Subgranular zone
SIRT	Sirtuins
SVZ	Subventricular zone
TBS	Tris-buffered saline
TET	Ten-eleven translocation protein
TNBC	Triple negative breast cancer
TP53	Tumor protein 53
YÜDETAM	Yeditepe Üniversitesi Tıp Fakültesi Deneysel Araştırmalar Merkezi

1. INTRODUCTION

1.1. CALORIE RESTRICTION

Calorie restriction (CR) is a non-genetic dietary intervention that could prolong life expectancy and maximize quality of life without causing malnutrition. It is basically limiting food intake to that which an organism would consume if given free access to nutrients. The life expectancy increases in direct proportion when the amount of CR is increased however when this level reaches the critical rate (around 55%) that affects vital activities, a rapid decrease is observed in life expectancy [1].

In the CR application, besides the amount of restricted calories, the types of restricted calorie sources and the restriction duration are also important [2]. In this context, mice on a low protein high carbohydrate diet ad libitum (AL) for 8 weeks showed similar improvements in insulin, glucose and lipid metabolisms as the 40% regular calorie restricted group [3].

Researchers have begun to look into new, more approachable types of CR because of the possible health benefits, such as chronic calorie restriction (CCR) and intermittent calorie restriction (ICR), which means application of CR followed by AL in a cyclic manner [4]. Recent studies have focused on comparing these two different CR methods (ICR vs CCR) [5–8]. Studies in mammals have reported a protective effect of CR against ageing and ageing-associated disorders such as obesity, cancer and neurological diseases in the past century [9–13] (Figure.1.1).

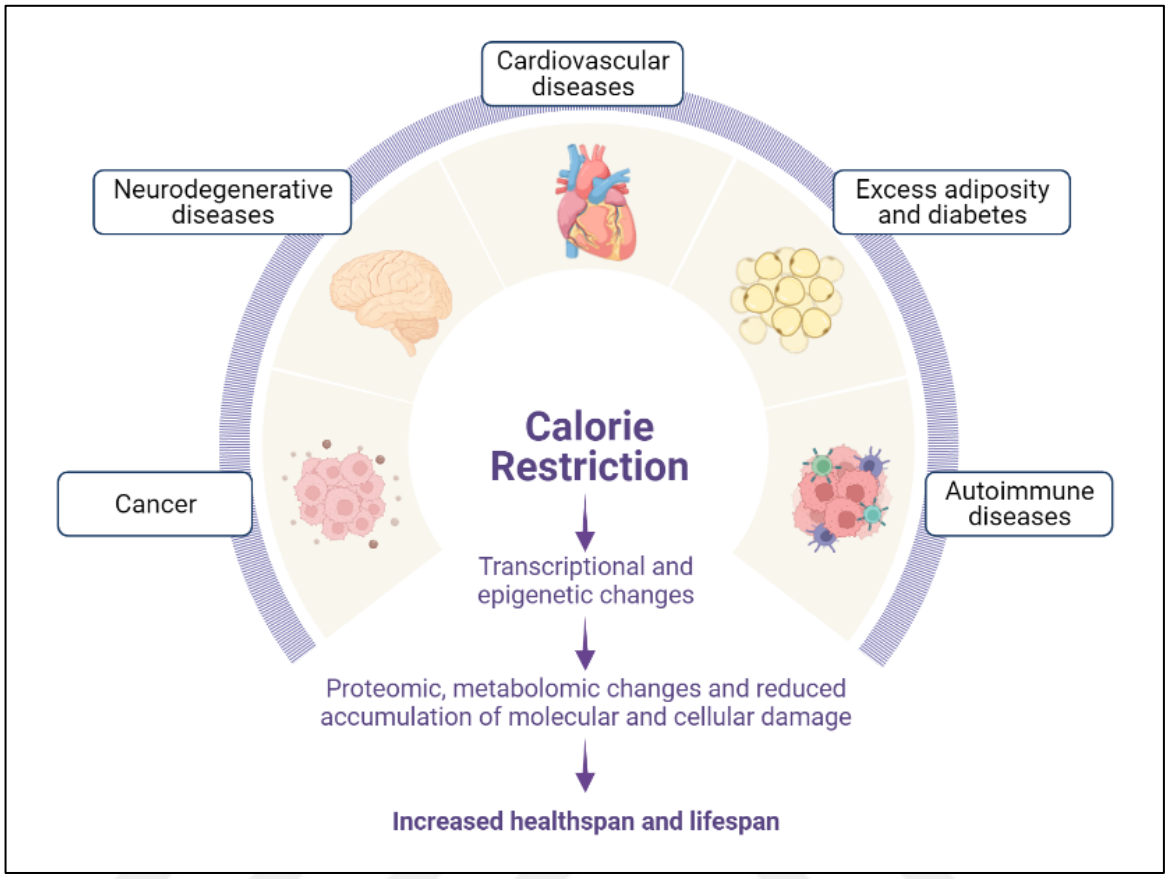


Figure 1.1. The effects of CR on lifespan

The anti-ageing and disease-preventing effects of CR were first announced by Osborne et al. in 1917, subsequently similar findings have been demonstrated across a wide and divergent species from unicellular organisms (yeast) to nematodes, invertebrates, and mammals [14–17]. Based on laboratory research on male and female rats, in nearly a century old study investigated by McCay et al. (1935) reported that CR with optimum nutrition (CRON) increase average and maximum lifespan [11]. In an another study with apoE deficient (apoE$^{-/-}$) both male and female mice, it was observed that 40% CR application for 16 weeks prevented vascular calcification and improved the amount of collagen and lipid ratio in the vessels [13].

Moreover, prevention of ageing-related neurogenesis reduction in the brain of C57BL/6 type mice which underwent 40% CR, was reported compared to the AL control group [10]. Furthermore preventive effects of CR in cancer development has also been showed in varied studies [18–21] For example, application of 20% CR for prevented lung metastasis by increasing tumor-fighting (CD4^{+}-CD8^{+}) immune cells in a 4T1 murine breast cancer model [12]. Dogan et al. showed in their newly published study that transgenic mice undergoing

chronic calorie restriction are less likely to acquire breast cancer and the outcome is regulated by miRNAs involved in the leptin and adiponectin pathways [22].

Many successful studies with model organisms like mice and rats have raised the question of whether CR could have a identical effect in non-human primates. In this context, one of the pioneering studies conducted with Rhesus macaques found a hazard ratio of 1.86 for animals that underwent 30% CR [23]. Their findings revial that the mortality rate of animals in the CR group was almost half of the ad libitum fed control group [23]. Similar to CR-dependent effects reported by previous studies which were conducted with a variety of mammalian models including rodents and primates [23–25] there is also a clinical trial outlining the constructive effects of CR on humans.

The most considerable study referring to effects of CR in humans is the CALERIE study, which is the first randomized clinical trial of CR started in 2002 [24]. In this study, 25% CR was applied to overweight, non-obese men and women for 6 months and followed up with exercise. The primary goals of CALERIE were to test the effect of long-term CR in humans and research even if CR would result in sustained metabolic adaptations, including reductions in core body temperature and decreased resting metabolic rate (RMR). In the same trial, authors also reported secondary outcome such as energy metabolism, cardiovascular risk factors, immune function, glucose and insulin parameters, endocrine response, qualify of life, psychological and cognitive functioning, physical activity, body composition (height, weight, and percent lean and fat mass), and nutrient intake [24]. The CALERIE trial also reported that CR resulted with reduced body weight, fat mass, blood insulin levels, and body temperature [25]. The another findings from the CALERIE trial were the improved cardiovascular factors including decreased triglycerides and total cholesterol in CR applied groups [25]. In addition, this study supports earlier conclusions about CR results in a decline in DNA damage [25].

Althought the helpful effects of CR in various physiological and patophysiological conditions have been known the exact mechanism(s) is/are yet to be clarified. In this context, many mechanisms such as transcriptional, proteomic and metabolomic modifications have been studied [26–28]. Furthermore, the effects of epigenetic mechanisms including DNA methylation in the preventive effects of CR have gained popularity in recent years [29,30]. In a study with budding yeast, when the sugar content in the culture media was reduced from 2% to 0.5%, positive correlations with survival were detected in genes related to

transcriptional regulation and ribosomal RNA production [31]. Miyamura et al. observed that 40% CR application for 8 weeks in early age (6-12 weeks) reduces the total 5-methyldeoxycytidine level in mouse liver tissue [32]. A recently published article reported a reduction in ageing-related methylation drift in rhesus macaques applied to 30% CR from 7 years of age until to death compared to the AL control group [33]. In this study authors claimed that methylation levels of rhesus macaques in CR group were delayed by 7 years compared that of AL group [33].

Taken together, the observations from mouse, non-human primate, and human studies support the roles of CR in healty ageing and ageing-related disorders. To clarify this phenomenon, the life-prolonging effect of CR in different model organisms has been tried to be explained in David Sinclair's unified theory of ageing [34]. In this work, David Sinclair argues that CR has an active role in the fight against stress, which plays an critical role in the survival of the organism [34] . In another study, it was investigated that DNA gap repair activity increased in corticol neurons of rats that were subjected to 40% CR for up to 30 weeks [35].

1.2. AGEING

Ageing is a natural progress that takes place in all living organisms as both structural and functional changes at the cellular and tissue level. These progressive changes lead to deterioration in body functions and ageing-related disorders over time. Age is the major risk element for chronic diseases, such as heart disease, cancer, and neurological disorders. While cardiovascular diseases (CVDs) related to ageing are vascular stiffnes and myocardial dysfunction ,also the most important factor in the appearance of neurodegenerative diseases [36,37].

Although ageing cannot be prevented, the rate of its progression could be delayed, and this may change the risk of developing diseases. In this context, telomere attrition, epigenetic alterations, cellular senescence, mitochondrial dysfunctions are some of the major changes altered by ageing at the cellular level [38–41] (Figure 1.2). All these changes lead to a slowdown in the body's self-repair and renewal mechanisms, and thus ageing occurs [42,43].

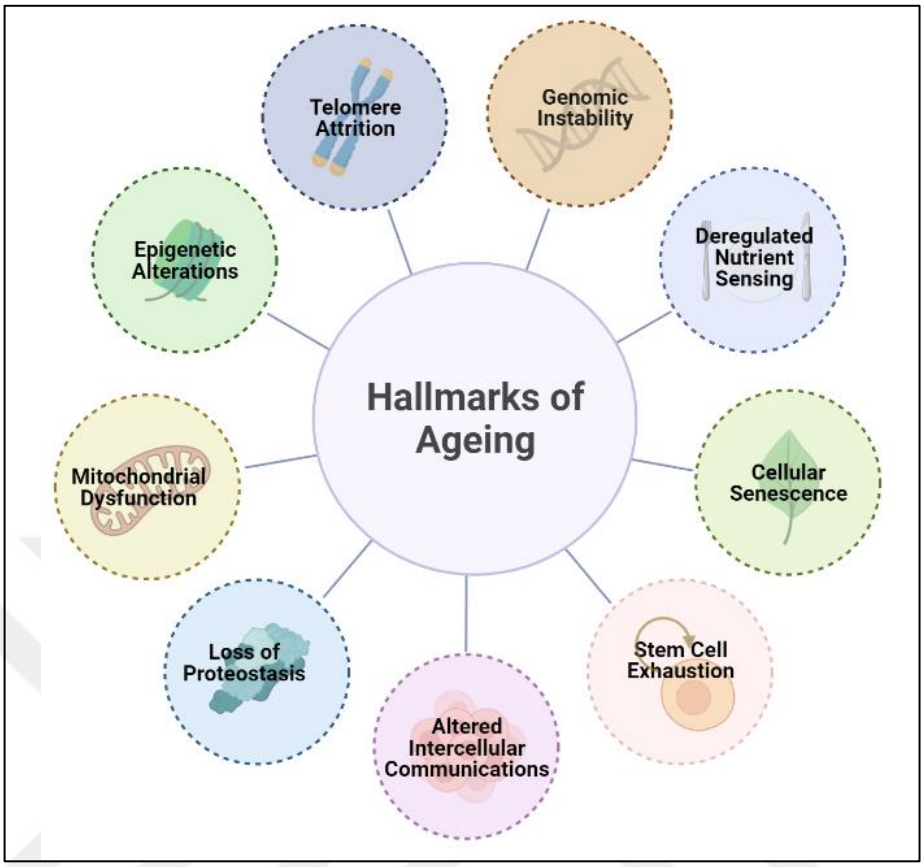


Figure 1.2. Hallmarks of ageing

Studies investigating ageing and the preventive effect of CR on ageing have been conducted for many years [44–48]. For example, the yeast replicative lifespan (RLS) is prolonged by nutritional administration by lowering the content of glucose in rich medium from the usual 2 % to 0.5 % or 0.005 % [49]. A study from the early 1990's conducted with fruit flies fed different amounts of yeast paste (1.5 and 0.15 mg respectively) reported in *Drosophila*, a decrease in yeast consumption results in an increase in lifespan. [50]. In a different study, Turturro et al. (1999) reported 40% CR application lowered body mass and increased longevity in a study conducted with approximately 60,000 animals from seven different genotypes, counting four mouse strains and three rat strains, and Fischer Brown Norway hybrid [51].

There are different factors that cause ageing and the well-defined biological pathways used in the emergence of these factors. Investigating these mechanisms and discovering new ageing-related biological advances is a huge step forward for the development of anti-ageing

treatments [52,53]. Although it is known that ageing occurs in every part of the body and in every system, the outcome it brings can be more easily followed and prevented in some systems and organs. The neuronal system is an example that we can easily follow the effects of ageing in the brain, and this usually occurs with morphological and functional changes in brain cells. The effects of ageing could take in many forms in the brain, but the most common is brain atrophy, which could result in reduced brain volume and decreased cognitive function.

Memory loss and dementia are common in ageing, since brain atrophy occurs especially in the prefrontal cortex and hippocampus, which are associated brain regions about memory [54]. The effect of ageing on the brain may also be due to changes in synaptic regulation, such as decreased synaptic plasticity and slowed neurotransmitter release [55]. In addition to the damage and death of neurons in the brain, the reduction in the number of new neurons to be produced is one of the negative changes in the brain caused by ageing [56]. Furthermore, proof for the presence of neural stem cells (NSCs) and adult neurogenesis in regions of the brain, such as cerebral cortex, hypothalamus, and white matter, has been found, and it has been proposed that diminished adult neurogenesis may play an critical role in neurodegenerative disorders, neural circuitry repair, and weight control [57–61].

Numerous signaling mechanisms have been shown to mediate and/or alter the anti-ageing benefits of CR [62]. Through genetic manipulation experiments in model organisms, many individual elements of these pathways have been verified as drug development targets such as FoxO, mTOR and sirtuins. Among these molecules, Sirtuin 1(SIRT1) is one of the most commonly investigated protein in ageing studies since it regulates food sensing, energy metabolism, and genomic stability [29,63–65]. Therefore we wanted to focus on this protein in the current thesis.

1.3. SIRTUINS

SIRTs are an evolutionarily conserved family of NAD+-dependent deacetylases that cut out acetyl groups from lysine residues on specific protein substrates**.** In mammals, seven sirtuins (SIRT1-7) have been found homologous to the Saccharomyces cerevisiae gene silent information regulator 2 (Sir2) [66]. Deacetylases include *Sirt1, Sirt2, Sirt3, and Sirt7*. Deacetylase, desuccinylase, and demalonylase activities have been discovered in *Sirt5. Sirt6*

possesses deacetylase, ADP-ribosyltransferase, and demyristoylase activity, according to reports [66]. *Sirt4* possesses at least an ADP-ribosyltransferase activity, despite the fact that its enzymatic activity is unknown [66]. NAD+ is necessary for all of these enzymatic functions of sirtuins, implying that sirtuins work as a sensor of the cellular energy state represented by NAD+ [67]. In the cells, their activity are also segregated. *Sirt3*, *Sirt4*, and *Sirt5* can only be found in mitochondria [68]. Recent studies have reported sirtuins play central roles in vital biological pathways such as DNA damage repair, telomerase activity, autophagy, circadian rhythm, cancer and many other mechanisms associated with ageing [66,67,69].

SIRTs are broadly conserved throughout life forms ranging from bacteria to humans [67,70,71]. The crucial significance of SIRTs as major elements of energy metabolism in all examined living organisms supports this gene expansion [72]. In Saccharomyces cerevisiae, sirtuins were found to be involved in the progression of ageing and lifespan. Yeast mother cells with an extra copy of the Sir2 gene have a 30% increase in replicative lifespan, however their lifespan is reduced by 50% when the gene is deleted or mutated [66]. Likewise, Caenorhabditis elegans with a higher dose of sir-2.1, the yeast Sir2 orthologue, have a 15–50% longer lifespan [73].

Mammals' SIRTs participate in a number of biological pathways, including age-related pathologies [74]. Furthermore, brain sirtuins have been shown to control a number of age-related pathological pathway s in energy homeostasis, cognition, emotion, and neurogenesis [68]. For example, in a *Sirt1*-knockout mouse, it was reported that the effects of CR could not be seen in the whole body without *Sirt1* expression, but especially in the brain. This result also announced that *Sirt1* plays an demanding role in phenotypic regulation in calorie-restricted mammals [68,75,76]. *Sirt1*-overexpressing transgenic mice, on the other hand, show CR-like traits or protective effects against metabolic problems caused by a high-fat diet and ageing [75,77,78].

Among the seven different sirtuin members, SIRT1 is the most comprehensively studied one which plays role in ageing and energy expenditure by regulating several cellular mechanisms such as stress response, cell survival, DNA repair, and circadian rhythm [78].

1.3.1. Sirtuin 1

The SIRTs are a family of mammalian class III histone deacetylases that are highly associated with lifespan and good health. SIRT1 is the most well-known and extensivelly researched protein in this family. It has emerging role in mitochondrial biogenesis and energy homeostasis[79].Due to its enzymatic activities, SIRT1 contributes to the growth of cancer, counting cardiovascular and neurodegenerative diseases [80–83].

SIRT1 is expressed and plays regulatory roles in many systems and organs in mammals including liver, heart, muscle and adipose tissue as well as in brain [71,84,85]. SIRT1 can be located in similar places in humans and mice and is responsible for similar functions, including the hippocampus, basal ganglia, and prefrontal cortex, which are parts of the brain responsible for the development of neurodegenerative diseases [86]. We can also understand that *Sirt1* and ageing are directly related from the decrease in the amount of SIRT1 in the hypothalamus with ageing, but this is not true for the amount of SIRT1 in all brain regions [87].

SIRT1's position in the cell is determined by tissue type, cell stress, and the cell's molecular mechanism. SIRT1 has been found in both the nucleus and the cytoplasm, and regulatory mobile elements are responsible for it's subcellular localization [88–90]. Both cytosolic and nuclear localization may be seen throughout the mouse brain and spinal cord, while immunocytochemical and Western blot investigations show that SIRT1 is mostly nuclear [86].

There are localization signals and export signals responsible for the nuclear localization of SIRT1, and the comparison of the strengths of these signals determines the localization of the protein in the cell. Of course, this varies with each cell type or environmental signal[88]. Numerous proteins in the cell are regulated by SIRT1 in the body, and these proteins are mostly associated with cellular ageing and metabolism. For example, SIRT1 is responsible for the deacetylation of the p53 protein, the guardian of the genome, which is associated with ageing, DNA repair and cancer formation in the cell [70,91,92] (Figure 1.3) .

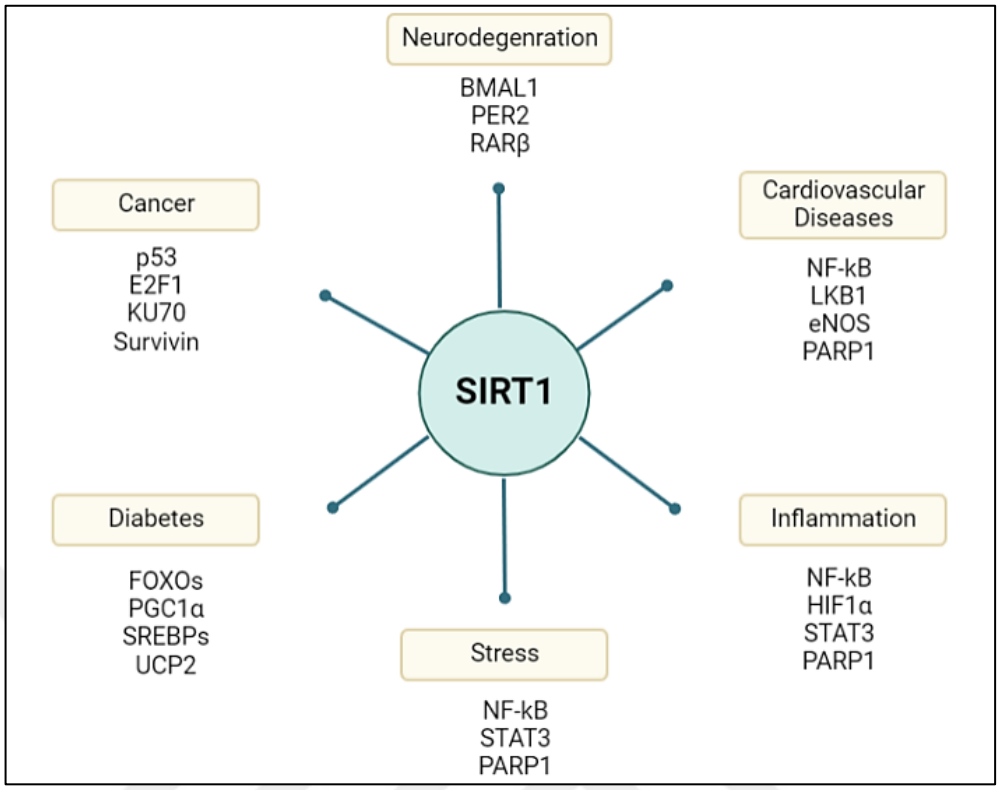


Figure 1.3. SIRT1 related cellular pathways

Elevated activity, enhanced glucose and insulin metabolism, decreased IGF-I signaling, and reduced reproduction are all characteristics of CR [93]. The lowering effect of CR on the IGF-1 signaling pathway and the correction of behavioral disorders were not observed in whole body and brain-specific *Sirt1* knockout mice [65,76]. Furthermore, looking at mice that overexpressed *Sirt1* without appliying CR were observed to show improvements in calorie-restricted mice. For example, lower cholesterol and glucose tolerance, weight loss, and lower insulin levels [94]. Uncontrolled activity, on the other hand, could cause tissue deterioration and, eventually, chronic diseases.When SIRT1 is inhibited, there is an increase in the amount of p53 in the cell and this increase causes a premature senescence-like phenotype, while overexpression reverses this stress-related senescence-like phenotype [95,96].

Due to these impressive properties of *Sirt1*, new ways to increase its effect in the body are sought. Resveratrol, a short polyphenol discovered through an in vitro screen, was the first SIRT1 activator to be intensively researched [97]. Resveratrol advances insulin sensitivity, inhibits tumorigenesis, lowers inflammation, boosts cardiovascular health, and protects against neurodegenerative illnesses, among other actions that are compatible with SIRT1

activation [98–101]. Furthermore, resveratrol imitates the transcriptional patterns associated with CR [44,102] and protects obese animals from early death [101]. But its action mechanism is still controversial because resveratrol can activate SIRT1 only on the use of fluorescent substrates and it has other targets in mammalian cells rather than SIRT1[103,104].

Despite there are some studies about the effects of CR on *Sirt1* mRNA and protein expression levels in ageing mouse brain, to our knowledge there is no study which reports the epigenetic modifications of *Sirt1* in brain.

1.4. EPIGENETICS

With no modification to the DNA sequence, epigenetic modifications could result in heritable phenotypic changes. The major categories of epigenetic modifications are thought to be key regulators of the ageing process [38,39,41,42]. Epigenetic changes are effective from embryonic development to the end of life, and epigenetic dysregulations arising from gene clusters can cause diseases [105]. For example, in a mouse model, transcriptional irregularities have been shown to affect synaptic plasticity and neural dysfunction, leading to neurodegeneration. It has been revealed that the reason for these transcriptional changes is epigenetic changes in chromatins that correlate with impaired synaptic plasticity [106]. Also, studies on the effects of epigenetic mechanisms on cancer development revealed a catalog of recurrent somatic mutations in several epigenetic regulators has been generated by whole-genome sequencing in a wide variety of malignancies [107].

The reason why epigenetic mechanisms have aroused a lot of interest lately is that they are mostly recyclable and flexible [40,108]. Environmental elements that have been linked to aging can delay some of these epigenetic alterations. Importantly, research in model organisms have shown the causal role of chromatin remodelers, transcriptional networks, noncoding RNAs, histones themselves, and chromatin modifiers of histones and DNA in determining lifespan [109]. DNA methylation accounts for the majority of recent studies, so we focused on the effect of DNA methylation on *Sirt1* expression in our study.

1.4.1. DNA Methylation

DNA methylation is typically involvement of methyl group addition covalently to cytosine which is followed by guanine via a phosphodiester bond (CpG dinucleotide) by methyltransferases [110–112]. CpG dinucleotides are mostly located in the regulatory region of the genome and these are primarily promoter or first exon of the gene. When there is high concentration of CpG sites (CpG dinucleotides) this area referred as CpG islands [110–115]. CpG islands are mostly unmethylated so transcription factors can bind to regulatory regions and gene transcription can start. But when its methylated the gene transcription is inhibited because of blocked transcription[110] (Figure 1.4).

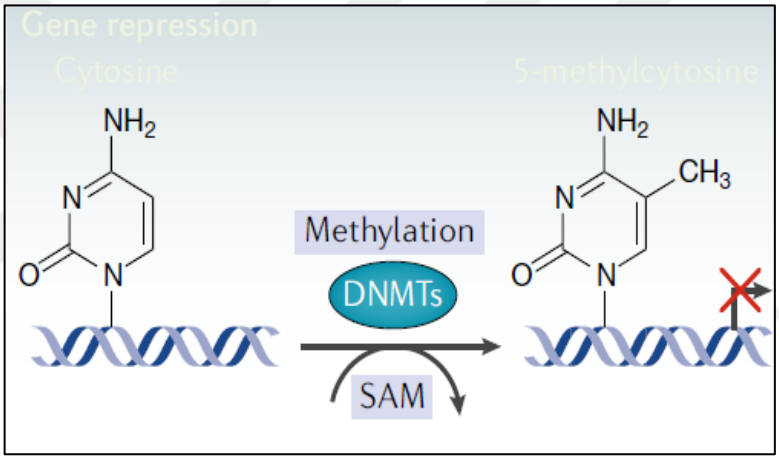


Figure 1.4. Mechanism of DNA methylation

[116]

DNA methylation has a variety of activities in mammals, including heterochromatin formation, imprinting, and X-chromosome inactivation[112]. Differences in methylation levels over time, on the other hand, can have negative consequences. For example, since hypermethylation in the regulatory regions of tumor suppressor genes will lead to a decrease in gene expression, cancer development and progression is promoted by this negative regulation. On the contrary, hypomethylation in oncogenes will cause an increase in gene expression and thus accelerate tumor development [117–122]. Studies suggest that genomic instability which is related to DNA methylation occurs with ageing. But not every region of the genome is hypomethylated. Promoter regions, which play an critical role in gene expression, are often hypermethylated with ageing.

Certain types of diet and CR have the effect of regulating epigenetic changes that are altered with ageing, and DNA methylation is a sensitive epigenetic mechanisms to environmental changes. It is now well accepted that leading a healthy lifestyle and eating a low-calorie diet can help you live longer. The fundamental mechanisms, however, are still being researched. Recent research has discovered changes in DNA methylation in obese individuals and presented epigeneticly important gene regions that mediate the response to low-calorie supply. According to the study, SIRT1, whose concentration increases with CR, changes in relation to DNA methylation changes in the cell [123]. In another experiment based on cell culture, SIRT1 expression decreases with ageing in living organisms and there is a correlation between DNA methylations events and SIRT1 expression levels [124].

SIRT1-mediated actions may prolong life by preserving epigenomic stability and a correct methylation pattern. Furthermore, the steady failure of DNA methylation with ageing may be linked to decreased DNA methyltransferase activity (DNMTs). SIRT1 regulates DNMT1, which is involved in DNA methylation. SIRT1 deacetylates DNMT1, enhancing its DNA methyltransferase function and transcriptional repressment [125]. In this thesis, DNA methylation levels of *Sirt1* was measured and compared in animals with and without mammary tumor development since role of *Sirt1* has also been shown in cancer.

1.5. CANCER

Cancer is a malignant mass that occurs when cells in any tissue of the body begin to grow uncontrollably, and they can affect the whole body with their ability to metastasize [126]. Cancer cells have common distinctive features and the transformation of healthy cells into cancer cells in the body is called tumorigenesis[127]. In addition to these hallmarks, two new hallmarks (cellular energetics and evaded immune destruction) as well as two new features (genome instability and tumor-promoting inflammation) have recently attracted attention [128]. Several internal and external risk factors contribute to tumorigenesis (cancer production). Genetic and epigenetic changes, Mutagenesis, instability in DNA repair pathways, and hormone abnormalities are all intrinsic risk factors[129–133]. Another characteristic of aging in multicellular creatures with regenerable (i.e., repairable or regenerative) tissues is gain-of-function modifications that allow cells to multiply uncontrollably (hyperplasia). Additionally, these modifications enable cells to develop

characteristics that improve their capacity to proliferate, move to, and colonize ectopic places, endure adverse tissue conditions, and resist immune system attack due to genetic instability. Of course, these traits are characteristics of cancers that are deadly [127]. Considering the hormonal effects of ageing, the effect of CR and ageing on breast cancer in female mice is quite interesting.

1.5.1. Breast Cancer

One of the most prominent cancer in the world is breast cancer, and it is the second most fatal malignancy across all females globally [133]. In the development of breast cancer , several complicated systems, internal and extrinsic risk factors all play a role. Genetic and epigenetic abnormalities are the most important of these intrinsic risk factors. It is widely known that cancer development increases with ageing. One of the most effective things in this relationship is the mutations and senescence that accumulate with ageing [134]. The accumulation of mutations in the DNA are one of the best examples of this common risk factor, and these mutations trigger cancer development by causing changes in the expression of tumor suppressor genes and oncogenes. BRCA1 and BRCA2 gene mutations [135].

For breast cancer studies, transgenic mouse models that cause mammary tumour development in mice were used [136,137]. It is frequently stated that TGFa and EGF act as powerful mitogens in a variety of epithelial cell systems [138]. For this reason, this mouse model was used in this thesis to observe the effects of mammary tumour development.

A target protein deacetylated by Sirtuins is p53, and activation of Sirtuins causes p53 to lose its tumor suppressive effect. Loss of function of the p53 gene results in cancer initiation, progression, and invasion of the cancer. SIRT1's role in cancer appears to be contradictious. SIRT1 upregulation has been reported in numerous malignancies as compared to normal tissues [136–138], while SIRT1 downregulation has been shown in a different tumors, including breast cancer [139].

SIRT1 has a variety of functions based on its subcellular localization (both nucleus and cytoplasm) and expression in different tissues, therefore it's natural to expect it to have a variety of effects on different types of cancers. According to a research, SIRT1 may have a key function in hypoxia, hence it is vital in a hypoxic tumor environment [140].

Understanding the role of SIRT1 in reducing p53 activity in a localization-dependent manner could bring us one step closer to regulating ageing and carcinogenesis. We have previously mentioned that SIRT1 suppresses p53, an anti-apoptotic mechanism it uses to inhibit the cell cycle and promote tumorigenesis. But p53 suppression is not the only anti-apoptotic mechanism SIRT1 uses [140]. SIRT1 also deacetylates FOXO members (FOXO1, FOXO3, and FOXO4), causing pro-apoptotic genes to be repressed and stress-resistance genes to be upregulated [141].

Knowledge of the connections between SIRT1 and p53, as well as their related regulators, can help researchers manipulate and regulate the signaling pathway involved in the creation of innovative treatments for breast cancer and degenerative disorders [128]. Despite the fact that SIRT1 has been linked to a variety of cellular processes including senescence, ageing, proliferation, gene silencing, and prolonged lifespan, the interaction between SIRT1 and DNA repair in tumour and normal tissue is not well understood and needs to be further investigated.

1.6. AIM OF THE STUDY

In this thesis, DNA methylation, mRNA expression, and protein expression levels of SIRT1 in brain samples isolated from mouse that were subjected to different types of dietary interventions were examined. The focus of this thesis is to see the results of long-term application of CR on DNA methylation of SIRT1 in the brains of ageing mouse, to determine the effects of CR on the brain and to see the anti ageing results of CR in brain methylation . Also, comparing DNA methylation profiles in brain of MT+ and MT- mice to observe the changes of MT development on the brain in MMTV-TGFα mice at week 81/82. The hypothesis is that CR application increases SIRT1 expression in the brain and the regulatory mechanism behind this is DNA methylation.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Equipment

Table 2.1. Equipment used in experiments

#	Brand	Item	Catalog No
1	ThermoScientific	FinnPipette Micropipettes (1000µl, 200µl, 20µl, 10µl, 2µl)	4642010, 30, 60, 80, 90
2	MYMED	Sterile Scalpel Tip 10	TIB-00401
3	Parafilm M	Parafilms	05801001
4	QIAGEN	PyroMarkQ24 Plate	979201
5	QIAGEN	PyroMarkQ24 Cartridge	979202
6	QIAGEN	PyroMarkQ24 Vacuum Prep Troughs	979206
7	QIAGEN	PyroMarkQ24 Plate Holder	9022273
8	QIAGEN	PyroMarkQ24 Vacuum Prep Filter Probe	979010
9	Corning	96 well plate	3596
10	GE Healthcare	PVDF blotting membrane	10600023
11	ThermoScientific	Western filter filter paper	84784
12	abcam	Bis-Tris Precast Gels-RunBlue	Ab270467

2.1.2. Instruments

Table 2.2. Instruments used in experiments.

#	Brand	Item	Catalog No
1	Beckman Coulter	Microfuge 16	392244
2	ThermoFisher	NanoDrop 2000	ND-2000
3	Haier Biomedical	Ultra-Freezer -86^{o}C	DW86L628
4	QIAGEN	PyroMark Q24 Vacuum Workstation	9001516
5	QIAGEN	PyroMark Q24 Advanced Software	9022779
7	BioRad	CFX96 Touch RT PCR	1855196
8	BioTek	EPOCH Spectrophotometer	21081918
9	BioRad	ChemiDocTM XRS imageing system	1708265
10	BioRad	Mini-Protean Tetra System	1658000
11	Novex	X cell SureLock	EI0001

2.1.3. Chemicals

Table 2.3. Chemicals used in experiments.

#	**Brand**	**Item**	**Catalog No**
1	Merck	TRIS Buffer, pH 8.0	648314
2	GE Healthcare	Streptavidin Sepharose® High Performance	17-5113-01
3	QIAGEN	PyroMark Denaturation Solution	979007
4	QIAGEN	PyroMark Wash Buffer concentrate	979008
5	Thermo Scientific	RIPA buffer	89900
6	BioRad	DC Protein Assay	500-0113/0114/0115
7	Sigma	Trizma base	1001681492
8	Invitrogen	UltraPure Glycine	15527-013
9	Oxoid	Skim milk powder	LP0031
10	BioFroxx	Tween 20	1247ML500
11	ThermoScientific	Super Signal West Femto	34096
12	ThermoScientific	0,5 M EDTA Solution (10X)	1861283
13	ThermoScientific	Halt Protease and Phosphatase Inhibitor(100X)	1861284
14	ThermoScientific	Pierce RIPA buffer	89900
15	Novex	Bolt SDS Running Buffer (20X)	B000202
16	Novex	LDS buffers(4X)	B0007
17	Novex	Sample Reducing Agent(10X)	B0009
18	BioLabs	Gel Loading Dye (6X)	B7025S
19	BioLabs	Quick load 100 bp DNA Ladder	S:N05515
20	Intron	RedSafe Nucleic Acid Staining (20.000X)	21141
21	BioLegend	Prime-Step Prestained Protein Ladder	773302
22	Sigma	Agarose	A9539-100G
23	Sigma	Bovine Serum Albumin	A6003-5G
24	Gibco	DPBS	14190-094

2.1.4. Kits

Table 2.4. Kits used in experiments.

#	**Brand**	**Item**	**Catalog Nu**
1	QIAGEN	DNaesy Blood and Tissue Kit	69504
2	QIAGEN	EpiTect Bisulfide Kit	59104
3	QIAGEN	EpiTect Whole Bisulfitome Kit	59203
4	QIAGEN	PyroMark PCR Kit	978705
5	QIAGEN	PyroMark CpG Assays	978746
6	QIAGEN	PyroMarkQ24 Advanced CpG Reagents	970902

2.2. ANIMALS

In this thesis, MMTV-TGFα (C57BL/6) positive male mice supplied from Hormel Institute Medical Research Center and bred with TGFα negative female mice (C57BL/6) to produce heterozygote MMTV-TGFα female pups. These mice have been identified as transgenic mice that are prone to developing breast cancer in the postmenopausal phase. It has been revealed that MMTV-TGFα mice show cancer development characteristics similar to human breast cancer [142]. Overexpression of EGFR/ErbB cascade member TGFα is a important factor in the emergence of MT development in MMTV-TGFα mice[143].

2.3. EXPERIMENTAL SETUP

Ten weeks old female MMTV-TGFα mice were randomly assigned to ad libitum (AL), chronic, and intermittent calorie restriction (CCR and ICR) diets. As a pellet feed, AltrominTPF1414 from Kobay A. (Ankara, Turkey) was employed. Water was freely available to all animals.The feeding regimens were used to determine food availability for MMTV-TGFα mice throughout the study. Ten weeks-old female MMTV-TGFα mice were randomly assigned to ad libitum (AL), chronic, and intermittent calorie restriction (CCR and ICR) diets. AL group of mice had unlimited access to food all the time. CCR group of mice were exposed to 15% restriction throughout the experiment. Furthermore, in the ICR group, one week of 60% CR was followed by three weeks of AL. The ICR group was separated into two groups weeks based on dietary regimen. ICR group mice sacrificed at the end of AL feeding were referred to as ICR-refeeding (ICR-RF), while mice sacrificed at the end of CR feeding were referred to as ICR-restriction (ICR-R). All animals were fed overnight one day before sacrificing. Animal food consumption was recorded on a daily basis.

2.4. SAMPLE PREPARATION

Five brain tissue out of each dietary intervention and five MT positive mice brain tissues were taken from the freezer and their weights were recorded. Samples were then sliced and inserted in 1.5 ml tubes. To eliminate from hair, blood, residues, and debris, samples were washed three times with 500 µl of sterile PBS (pipette up and down). On each sample, 0.5

mm zirconium bead was placed. PBS buffer twice the sample weight was added to each microcentrifuge tube; however, if the volume was over 300 µl, extra PBS was poured after homogenization and mixed to achieve a homogenous mixture.

For three minutes, samples were homogenized. Following the run, samples were examined and centrifuged at maximum RPM for 10 min at 4°C to discard beads. After mixing the samples, 100 µl were discarded to fresh 1.5 ml centrifuge tube for DNA isolation.

Table 2.5. Number of brain samples were collected at determined weeks and diets

Diet	**Week 10**	**Week 49/50**	**Week 81/82**
AL	5	5	5
CCR		5	5
ICR-R		5	4
ICR-RF		5	5
MT developed mice brain	-	-	5
Number of Mice	**5**	**20**	**24**

2.5. MEASUREMENT OF DNA METHYLATION IN MOUSE BRAIN

2.5.1. DNA Isolation

The genomic DNA (gDNA) was isolated from roughly 50 µl of brain homogenate with the Qiagen DNeasy Kit in accord with manufacturer's protocol. 180 µl buffer ATL, 20 µl proteinase K, and 50 µl brain homogenate were heated at 56°C for 3 hours (shaking at 300 rpm). After that, 200 µl AL buffer was poured to the sample and heated at 56°C for 10 min. 200 µl of cooled ethanol (96-100%) was poured, and the mix was transferred aseptically into a spin column inserted in a 2 ml collecting tube. After moving the column to a brand-new collecting tube, 500 µl of Buffer AW1 has been poured. After 1 minute of centrifugation at 8000 rpm, a wash with 500 µl Buffer AW2 was performed, followed by another 3 minute centrifugation stage (14,000 rpm). 100 µl Buffer AE was poured to spin column for elution and incubated for 1 minute at RT. Finally, the spin column was centrifuged to elute gDNA from column. The Nanodrop Spectrophotometer was used to determine purity and quantity of DNA.

2.5.2. Bisulfite Conversion

First, DNA was bisulfite-treated in accordance with the manufacturer's protocol using the Epi-Tect Bisulfite Conversion Kit. Bisulfite treatment changed all non-methylated cytosines to uracil, then non-methylated cytosines in the following PCR product turned into thymine, resulting pyrosequencing of the PCR product to lead to cytosine methylation identification. For each sample, 1000 ng of gDNA was bisulfite-treated. At the start of the experiment, 85 µl Bisulfite mix, 35µl DNA protect buffer, RNase-free water, and 1000 ng DNA were thoroughly mixed in the PCR tubes, and bisulfite conversion has been done with thermal cycler under the conditions specified in the Table 2.6.

Table 2.6. Thermal cycler conditions for bisulfite conversion

Step	Time	Temperature
Denaturating	5 min	95ºC
Incubating	25 min	60ºC
Denaturating	5 min	95ºC
Incubating	85 min	60ºC
Denaturating	5 min	95ºC
Incubating	175 min	60ºC
Holding	∞	20ºC

After adding 560 µl Buffer BL, the mixture was placed to EpiTect spin columns and centrifuged for 1 min at 13,000 rpm. Following a 15-minute incubation with 500 µl Buffer BD at RT, 500 µl Buffer BW was poured to column. After another wash with 500µl Buffer BW, the spin column was incubated for 5 minutes at 56°C in a heater with an open cap into 1.5 ml microcentrifuge tube. To remove the pure DNA, 20 µl Buffer EB was poured over the each membrane and spin column was centrifuged at 13,000 rpm for 1 min.

2.5.3. Whole Bisulfitome Amplification

To achieve uniform amplification over the whole bisulfite-converted genome, the EpiTect Whole Bisulfitome Kit was used. The following steps were taken to amplify 2 µl bisulfite

converted DNA in 29 µl EpiTect Whole reaction buffer and 1 µl DNA Polymerase: 8 hours at 28°C and 5 minutes at 95°C . The amplification was done in a MultiGene Thermal Cycler.

2.5.4. PyroPCR

The PyroMark PCR Kit was used to perform PCR on bisulfite converted DNA under the given PCR conditions given in the Table 2.7.

Table 2.7. Thermal cycler conditions for PyroPCR

Step	**Time**		**Temperature**
Initial	15 min		95 ºC
Denaturating	45 cycles	30 s	94 ºC
Annealing		30 s	55 ºC
Extension		30 s	72 ºC
Final Extension	10 min		72 ºC
Holding	∞		4 ºC

Qiagen provided pre-designed *Sirt1* PyroMark PCR primers (Mm_*Sirt1*_01_PM PyroMark CpG assay) (Hilden, Germany). PCRs were made out of 12.5 µl PCR Mastermix 2x, 2.5 µl CoralLoad, 2.5 µl PCR primer set, and 2.5 µl DNA, made up to 25 µl with RNase-free water. The PCR reactions were performed in a MultiGene Thermal Cycler.

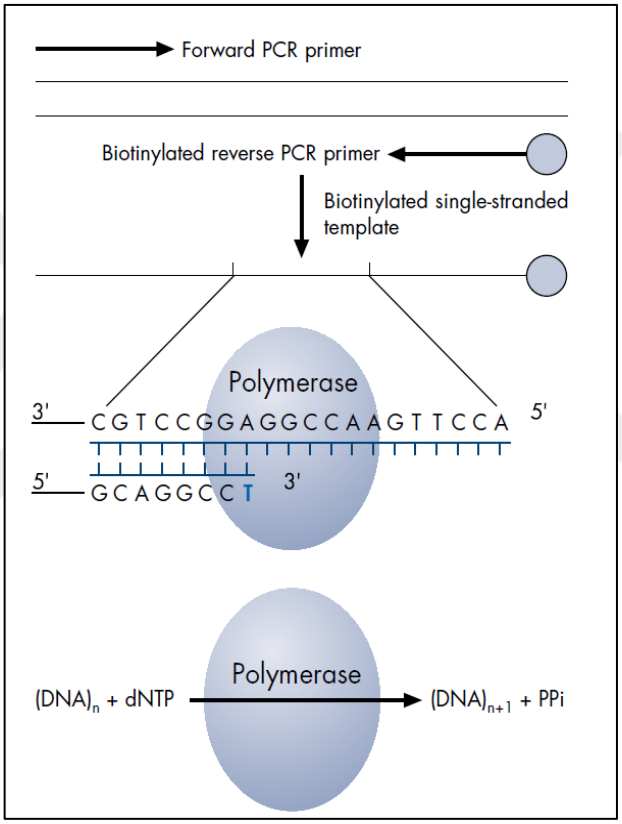
2.5.5. Agarose Gel Electrophoresis

Prior to pyrosequencing, to evaluate the quality of the product, 5µl of PCR product was tested on 1% agarose gel. One gel generally contained 4 g agarose, 8 ml of 50 x TAE buffer, and 400 ml of distilled water. The solution was melted in a microwave on maximum for 2 min, until the agarose was entirely dissolved and the liquid appeared transparent.

After melting, the solution was cooled to around 65 °C, and 20 µl of RedSafe was added, mixed thoroughly, and poured onto a prepared gel casting tray to cool until the gel looked milky and solidified. The sample slots were chosen based on the gel's intended use. Samples for electrophoresis were mixed with 6 x BX-loadıng dye and electrophoresis was performed

for 1 hour at 110 V in 1X TAE buffer. The isolated DNA fragments were seen in the Illumination chamber (Gel doc, Biorad) by illuminating the gel using UV light. Because the RedSafe is intercalating with the DNA, the DNA shows as bright bands under UV irradiation, while the remainder of the gel is black.

2.5.6. Pyrosequencing



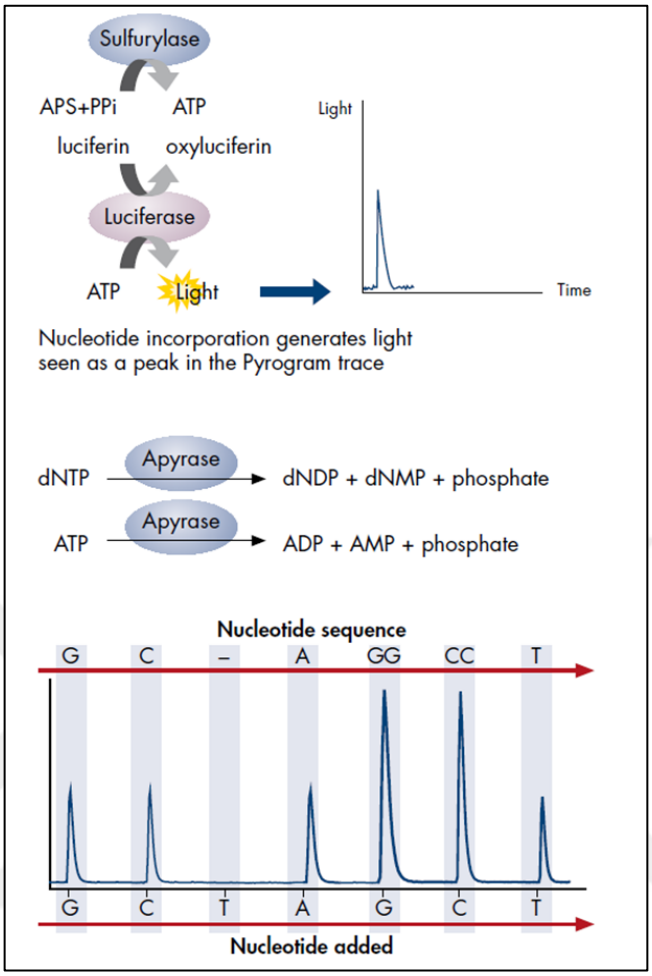


Figure 2.1. Mechanism of Pyrosequencing

To estimate % methylation, a pyrosequencing platform Q24pyrosequencer with PyroMark GoldQ24 Reagents was performed to the manufacturer's specifications. DNA strand was duplicated and new sequence that will serve as the guide for Pyrosequencing was biotinylated. After denaturation, the biotinylated single-stranded PCR product was extracted and let on to hybridize with a sequence primer. DNA polymerase promotes the insertion of the dNTP to the sequencing primer if it was complementary to the nucleotide in the guide sequence. Each pyrophosphate (PPi) release was equimolar to the level of free nucleotide. PPi was transformed to ATP by ATP sulfurylase when it was present. CCD sensors detect the light emitted by the luciferase-catalyzed process, which appears as a peak in the Pyrogram®. The amount of bases inserted decides the height of each peak (light signal). Apyrase destroys unincorporated nucleotides and ATP on a continual basis. Another nucleotide was inserted after the degradation is complete. The addition of dNTPs was done

in a proper sequence. The nucleotide sequence was inferred from the signals in Pyrogram as the complementary DNA strand lengthens during the procedure. The procedures performed in the laboratory for these reactions to take place are as follows.

First, the bead mix was prepared by mixing 40 µl of binding buffer, 18 µl of water and 2 µl of beads, and 60 µl per well was added to the 24-well PCR plate. 18 µl of the corresponding PCR output was placed in each well and the 24-well plate was closed with a transparent seal. It was mixed at 1400 rpm for 15 minutes until the vacuum station was ready for use. 25 µl of 1x sequencing primers in annealing buffer were placed on the PyroMarkQ24 plate. In the PyroMarkQ24 Advanced software, information about the wells of the samples was entered and it was controlled how much volume was loaded into the cartridge. After adding solutions to the cartridge, it was placed in the PyroMarkQ24 Advanced. The vacuum switch on the aspiration probe was opened and approximately 70 ml of distilled water was aspirated. With the vacuum on, the suction probe was inserted into the PCR plate wells, all the solution in the wells was carefully captured for about 15 seconds. Beads/samples were washed with 70% ethanol for 5 seconds. The tray with denatured solution was continued, washed for 5 seconds. Wash Buffer was aspirated for 10 seconds and tilted the suction probe beyond 90º. The probe was placed on the PyroMarkQ24 plate with no contact with the liquid. The vacuum was turned off and the lever on the PyroMarkQ24 plate was lowered. 70 ml of distilled water from the priming tray was aspirated. The plate was placed on a metal tray and heated 80 ºC , 5 minutes. The sequencing plate was placed on the machine, the lid was closed, and the run was started. The PyroMarkQ24 plate and the cartridge's unused contents were discarded by inversion when the run was finished, and the cartridge was then washed with deionized water.

2.5.7. Data Analysis

The PyroMarkQ24 software was used to analyse the pyrosequencing statistics. No template negative controls were used in any pyrosequencing run, and each assay sequence included bisulfite conversion controls to measure the effectiveness of the conversion process. The methylation analysis was carried out using the PyroMarkQ24 Advanced software for each variable CpG site, and the results were shown as a Pyrogram®. The methylation percentage for each variable position was determined using an Excel, and the data was exported.

2.6. MEASUREMENT OF MRNA EXPRESSION IN MOUSE BRAIN

2.6.1. RNA Isolation

Later, 50 µl of brain homogenates were taken into fresh tubes. Next, 600 µl of TRI Reagent was poured to the samples and vortexed vigorously for 1 min. Mixtures were chilled on ice for 5 min and centrifuged at RT at 16,000g. After transferring the supernatant to new microcentrifuge tubes, 600 µl of 96% ethanol was poured and vortexed into the mixture. Centrifugation was performed on 700 µl of this mixture that was placed into the Zymo Direct-zol MiniPrep Total RNA minicolumns and collecting tubes for 1 minute at maximum speed. Then, 450 µl RNA wash buffer was poured into tubes and centrifuged at RT, 16,000g. DNase I was applied to columns using a solution of 6 µl DNase I and 75 µl DNA Digestion Buffer. Then mix was placed directly into a matrix of columns, where they were incubated for 10 min at RT. Incubated minicolumns were centrifuged at maximum speed for 2 minutes and a spectrophotometer was utilized to measure RNA concentrations, and 1% agarose gel electrophoresis was utilized to control the integrity of total RNA.

2.6.2. RT-qPCR

The 20 µl reaction volume was then filled with enough distilled water, 4 µl of the 5x reaction mix, 1 µl of the reverse transcriptase, and 1 µl of the iScript reagents to perform reverse transcription on the 1 µl RNA sample. The mixture was heated to 25°C for 5 min , 42°C for 30 min, then 85°C for 5 min before being kept at 20°C for storage. All cDNAs were subjected to PCR for the sake of evaluating the cDNA's quality. The *Actb* gene was used for normalization. Primers were designed for *Sirt1* and *Actb*. PCR was accomplished by following the manufecturer's protocol. Quantitative PCR was then performed using an BioRad CFX96. The data were analyzed with BioRad CFX Manager software. Comparing the Ct values of the target gene with the Ct values of the Actb allowed us to determine the relative expression for each sample. The following primers were used: *Sirt1* forward primer, 5'-GCT GAC GAC TTC GAC GAC G-3'; reverse primer, 5'-TCG GTC AAC AGG AGG TTG TCT- 3'; *Actb* forward primer, 5'-ATG GAG GGG AAT ACA GCC C -3'; reverse

primer, 5’-TTC TTT GCA GCT CCT TCG TT -3’.qPCRs were performed in triplicate and repeated for confirmation.

2.7. MEASUREMENT OF PROTEIN EXPRESSION IN MOUSE BRAIN

2.7.1. Protein Extraction

For protein extraction, 10 µl of the brain homogenates from each group were employed. They were pipetted well after being thoroughly thawed on ice, and then centrifuged at 13.000 rpm for 10 minutes in fridge. The pellets were then dissolved in 100 l of radioimmunoprecipitation assay (RIPA) solution. The same amount of RIPA was added to the samples as PBS that was used for homogenization in the beginning. Each sample was then left on ice for 30 minutes while being lightly tapped every 10 minutes. After incubation, they were centrifuged for 5 mins, at 14.000 rpm, +4°C and supernatants were transferred into new eppendorfs.

2.7.2. DC Assay

The DC Assay was utilized to evaluate the concentration of soluble protein extracts. Bovine Serum Albumin (BSA, 1.5 mg/ml) was employed as a standard in concentrations ranging from 1.5-0.05 mg/ml. First, duplicates of 5 µl of BSA standards and unknown samples were loaded. Then, 25 µl of reagent A' (Reagent A' = A+S) was added to each well. At the final step, 200 µl of reagent B were applied to each well. Plate was shaken for 1 minute at 100 rpm on an orbital shaker before being placed in a dark environment for 15 minutes at RT. In the meantime, the BioTek microplate reader was allowed to calibrate. The existence of air bubbles on each well was examined prior to taking measurements, and any found were blown off. The absorbance were obtained at 750 nm. The absorbance of the blank was subtracted and a standart curve was plotted based on the net absorbance of standarts. Then, using a standard curve equation, the levels of the unknown proteins were determined.

2.7.3. Western-blot

Western-blotting is a common laboratory technique for separating a protein from a mixture and/or comparing the level of protein in different treatments. Optimization experiments were used to determine the level of protein injected into the wells. SIRT1 and the loading control GAPDH were both detected in the brain samples using 30 g total protein. Protein samples were diluted with ddH2O, then combined with 7,5 µl LDS sample buffer (4X) and 3 µl sample reduction agent (10X) in a total of 19.5 µl volume. Before loading into gel, samples were heated for 10 min at 95°C. After heating, they were waited on ice for 10 min. Ready-to-use SDS-PAGE gels were covered by gel casette and the short plate was facing inward as they were inserted into the electrode assembly. After checking for any linkages, the tank was loaded with 1X Running buffer. To eliminate any pollutants, the combs were gently removed without damaging the wells, and the wells were rinsed with 1X Running buffer. Each sample was carefully loaded to avoid the introduction of air bubbles. A protein ladder (Prime Step Broad Range Marker, BioLegend, Cat:773302) was loaded as 4 µl before the first sample.

As an electrophoresis system, Novex XCell SureLock was used. In order to ensure precise alignment, samples were run for 20 minutes at 100 V before being divided according to size. The voltage was subsequently raised to 130V, where the samples were run until the loading dyes reached the gel's end. For the activation, a polyvinylidene difluoride (PVDF) membrane was bathed in 100% methanol. Meanwhile, in the ice-cold 1X Transfer buffer, transfer equipment such as Whatman filter papers, gel holder casette, and foam peds awaited.

After the electrophoresis was completed, the short plates were taken out of the chamber together with the gel casette sandwiches.After discardig the gel, the transfer gel was assembled, from top to bottom: Black lid + Sponge + 2 filter paper + Gel + Membrane + 2 filter paper + Sponge + Clear lid. The transfer was done in the BioRad Mini Trans-Blot Cell Module (CA, USA). For one hour, the transfer was done at 85V. After gently opening the transfer sandwich, the membrane was washed with 1X TBST (Tris Buffered Saline-Tween 20) for 5 minutes at 100 rpm. Membranes were split into smaller parts based on where protein interests were found. More than one protein of interest could be tested per sample in this way. For blocking, membranes were incubated in the blocking solution on the shaker, 90 rpm for 1 hour. Blocking solution includes 0.5 g of milk powder and 10 mL of 5% TBST.

After the blocking step, they had an overnight incubation at 4°C with the primary antibody (Table 2.8). After primary body incubation, membranes were washed with 1X TBST three times. Then they were incubated with the secondary antibody (Table) for 1 hour at RT. Membranes were prepared for chemiluminescent detection by three 1X TBST washes after secondary antibody incubation.

Table 2.8. Antibody list used in the western blot experiments

Antibody name	Antibody type	Product code	Working concentration (v/v)	Host species
SIRT1	Primary	PA5-85921	1:1000	Rabbit polyclonal
GAPDH	Primary	M00227	1:5000	Rabbit polyclonal
IgG for SIRT1	Secondary	7074S	1:10000	Anti-Rabbit
IgG for GAPDH	Secondary	7074S	1:50000	Anti-Rabbit

The BioRad-ChemiDocTM XR 96 imaging system was used for Chemiluminescent detection. The tray was then cleaned with 70% ethanol. The chemiluminescent solution was made by mixing West Femto Peroxide buffer and West Femto luminol/enhancer solution once the imaging system was ready (1:1). The membrane was placed atop a black box and a chemiluminescent solution was applied to it. The membrane was placed on a tray after 1 minute of incubation and the protein of interest was visualized. It was optimised for intense bands by Image Lab 6.1 Software preventing signal saturation. Band intensity measurements were done through the Image Lab 6.1 Software. Each band was selected and the area under the curve was measured, then a normalization step was followed. Each antibody's band intensity was divided by the band intensity of the loading control (i.e., GAPDH).

2.8. STATISTICAL ANALYSIS

Microsoft Excel (Microsoft Office 2013) was used to collect the data for this study, and GraphPad Prism (version 9.0) was used for statistical analysis. Data are represented by the mean ± standard error of the mean (SEM). All of the data had a normal distribution. $P \leq 0.05$ were considered statistically significant. An analysis of variance (ANOVA) and Student T test were performed. *$P < 0.05$; **$P< 0.01$; ***$P < 0.001$.

3. RESULTS

In this thesis, we examined the change of *Sirt1* expression in the mouse brain due to different types of CR and the role of DNA methylation on *Sirt1* regulation in the whole mouse brain.

3.1. DNA METHYLATION LEVELS IN MOUSE BRAIN

3.1.1. The Effects Of CR on DNA Methylation Levels Of *Sirt1*

DNA methylation levels of 5 CpG sites were measured with pyrosequencing to understand the effects of different types of CR on the DNA methylations of *Sirt1* in the mouse brain. These 5 CpGs were located at the first exon and responsible for the transcriptional regulation of *Sirt1*. Average DNA methylation was calculated by averaging the 5 CpG regions. Following the validation of the normality, ANOVA tests were performed on DNA methylation data. One-way ANOVA test revelaed no significant change in CR application on the average DNA methylation levels of *Sirt1* at week 49/50 (Figure 3.1) and 81/82 (Figure 3.2) in CR. Following the validation of the normality, Analysis of Variances (ANOVAs) was performed on DNA methylation data. Two-way ANOVA test revealed no significant difference between the DNA methylation levels of 5 CpGs in *Sirt1* at week 49/50 (Figure 3.2 and (Figure 3.3) and 81/82 in CR (Figure 3.4). These findings propose that the *Sirt1* DNA methylation is be stable in the whole mice brain beside the dietary interventions and ageing.

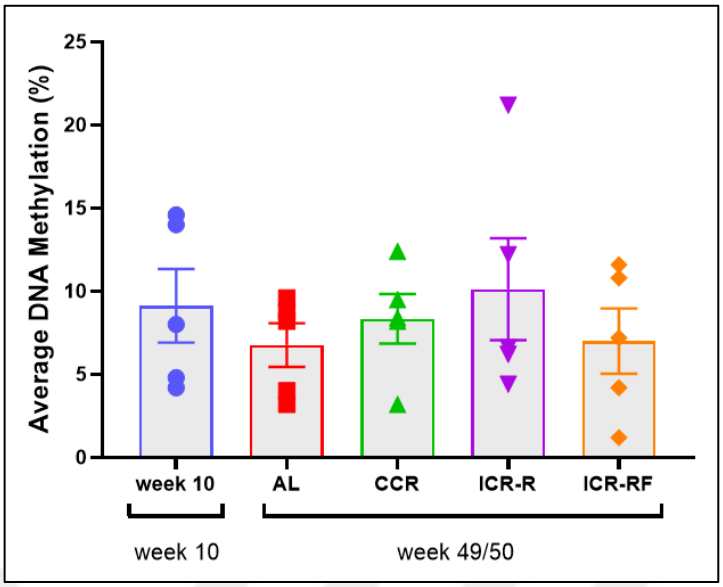


Figure 3.1. The effects of different types of CR on average DNA methylation levels of *Sirt1* in mice brain at week 10 (baseline) and 49/50. (n=5)

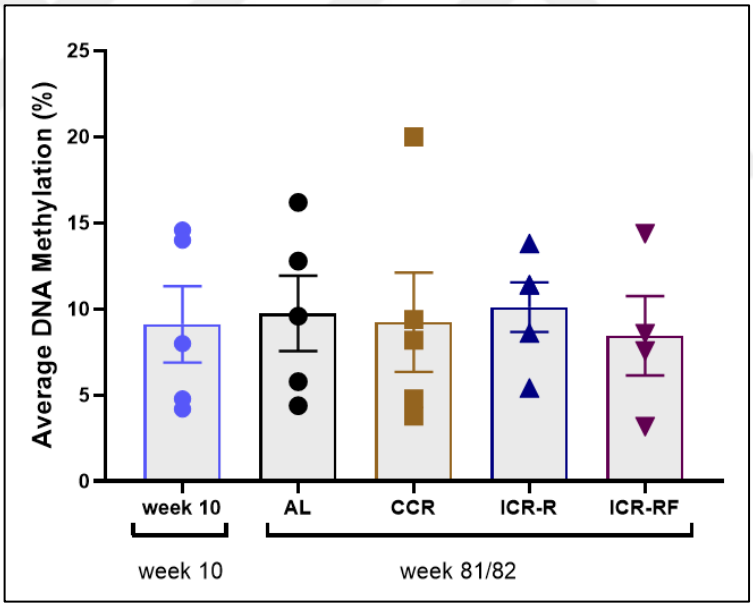


Figure 3.2. The effects of different types of CR on average DNA methylation levels of Sirt1 in mice brain at week 10 (baseline) and 81/82. (n=4 for ICR-RF, n=5 for other groups)

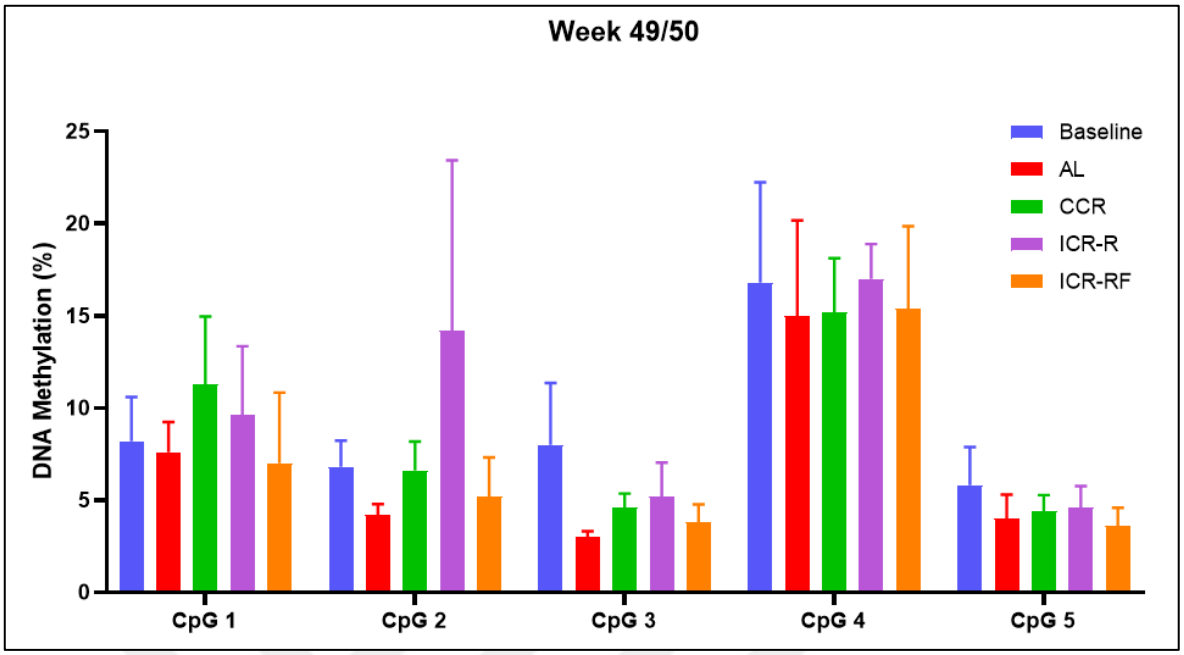


Figure 3.3. The effects of different types of CR on DNA methylation levels of Sirt1 in mice brain at week 10 (baseline) and 49/50. (n=5)

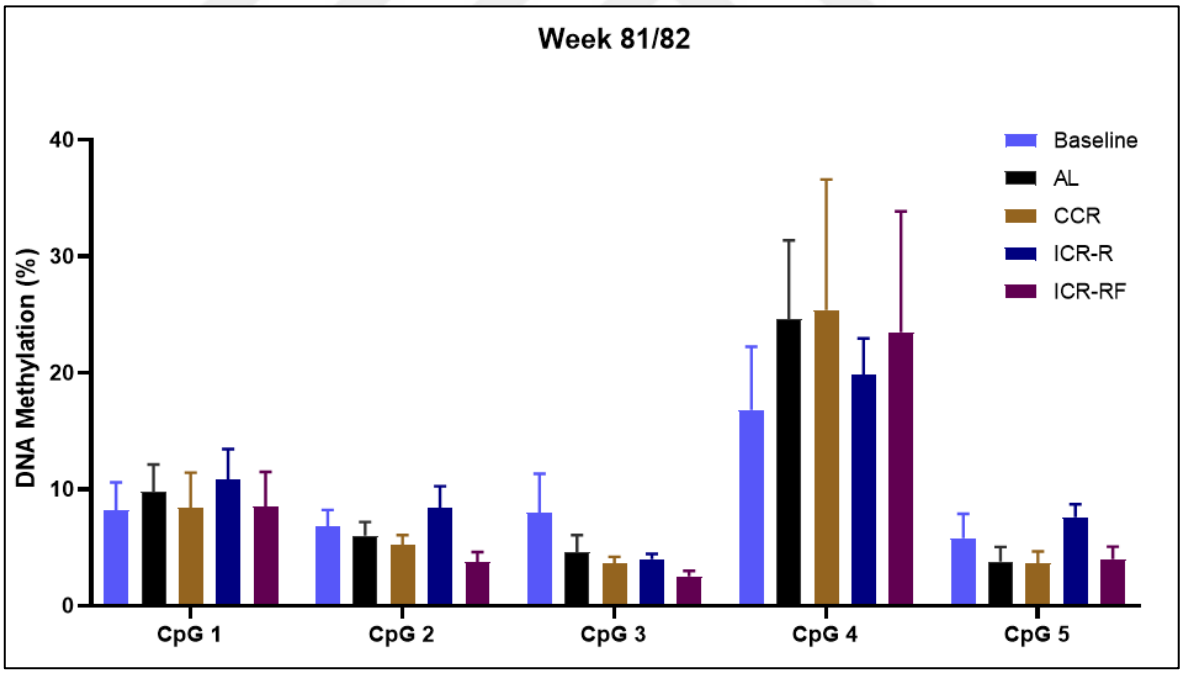


Figure 3.4. The effects of different types of CR on DNA methylation levels of *Sirt1* in mice brain at week 10 (baseline) and 81/82. (n=5 for week 10, AL, CCR and ICR-R, n=4 for ICR-RF)

3.1.2. The Effects Of Ageing on DNA Methylation Levels Of *Sirt1*

DNA methylation levels of 5 CpG sites were measured with pyrosequencing in AL-fed mice of 3 different ages to investigate age-related variation of *Sirt1* DNA methylation. Following the validation of the normality, ANOVA tests were performed on DNA methylation data. Average DNA methylation was calculated by averaging the 5 CpG regions. Following the validation of the normality, ANOVA tests were performed on DNA methylation data. One-way ANOVA test revealed no significant change on the average DNA methylation levels of *Sirt1* in different age groups (Figure 3.5). Two-way ANOVA tests revealed no significant change of duration on the DNA methylation levels of 5 CpGs in *Sirt1* on AL-fed mice at week 10, 49/50 and 81/82 (Figure 3.6). These results indicate that the *Sirt1* DNA methylation tends to be stable in the whole brain of AL-fed mice with advancing age.

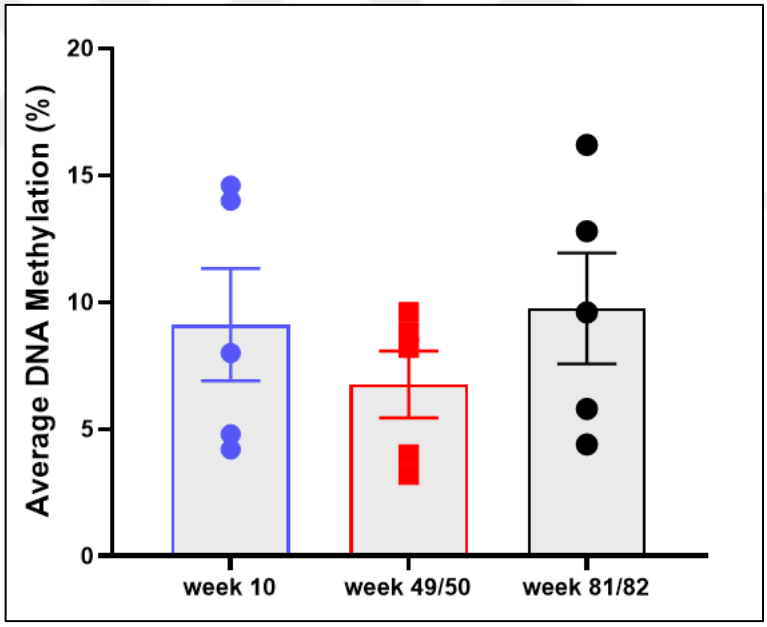


Figure 3.5. The effects of different types of CR on average DNA methylation levels of *Sirt1* in mice brain at week 10 (baseline), 49/50 and 81/82. (n=5)

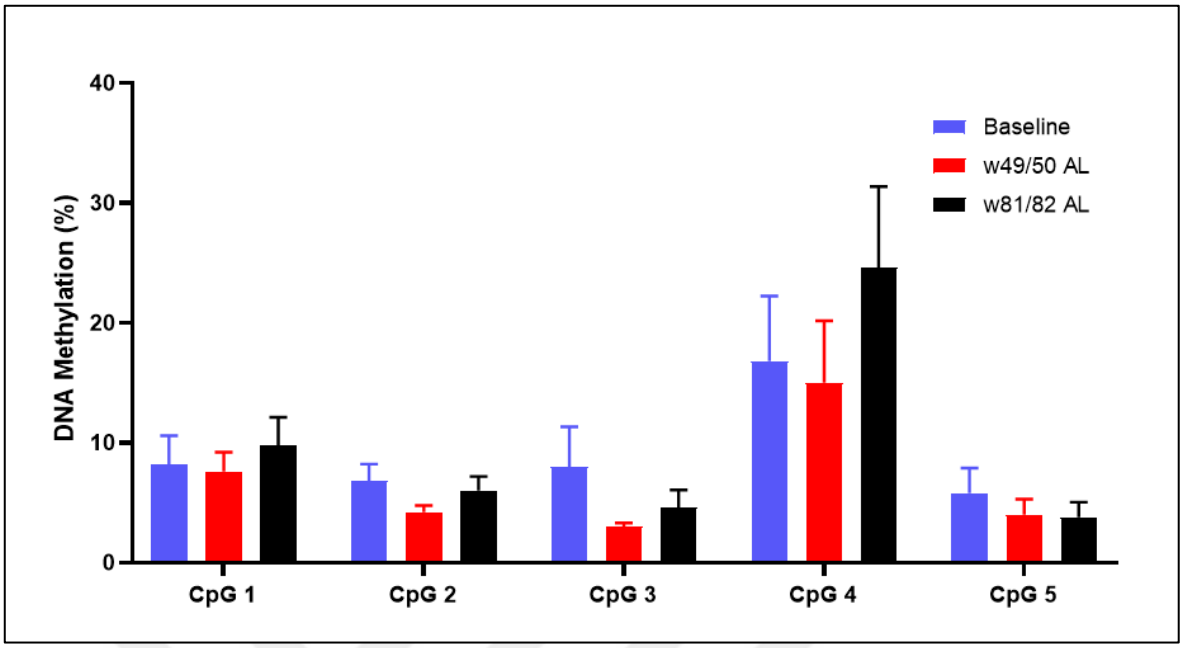


Figure 3.6. The effects of different types of CR on DNA methylation levels of *Sirt1* in mice brain at week 10 (baseline), 49/50 and 81/82. (AL: Ad libitum) (n=5)

3.1.3. The Effects of Mammary Tumour Development on DNA Methylation Levels of *Sirt1*

DNA methylation levels of 5 CpG sites were measured with pyrosequencing in mammary tumour developed and healthy AL-fed mice to understand the effect of MT development on *Sirt1* DNA methylation in the brain. Following the validation of the normality, ANOVA tests were performed on DNA methylation data. Average DNA methylation was calculated by averaging the 5 CpG regions. Following the validation of the normality, ANOVA tests were performed on DNA methylation data. One-way ANOVA test revealed no significant change in MT development on the average DNA methylation levels of *Sirt1* in MT- and MT+ groups (Figure 3.7). Two-way ANOVA test revealed no significant change on MT development on the DNA methylation levels of 5 CpGs in *Sirt1* at week 81/82 (Figure 3.8). These results indicate that the *Sirt1* DNA methylation tends to be stable in the whole brain of AL-fed mice with MT development.

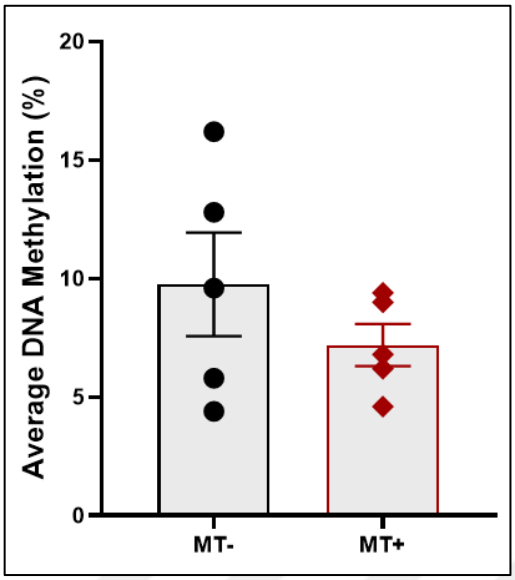


Figure 3.7. The effects of MT development on average DNA methylation levels of *Sirt1* in mice brain at week 81/82. The brain tissue which was taken from mammary tumour developed mice were designated as MT+, not developed mice were designated as MT-. (n=5).

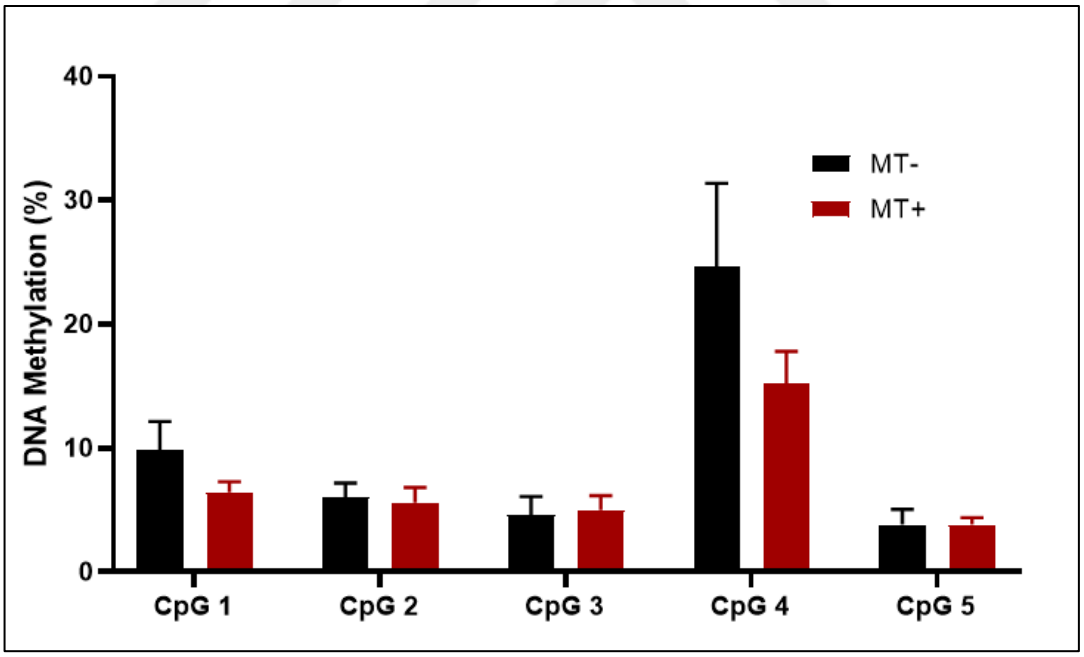


Figure 3.8. The effects of MT development on DNA methylation levels of *Sirt1* in mice brain at week 81/82. The brain tissue which was taken from mammary tumour developed mice were designated as MT+, not developed mice were designated as MT-. (n=5).

3.2. GENE EXPRESSION LEVELS IN MOUSE BRAIN

The DNA methylation performs a regulatory role in regulating gene expression and usually causes inhibition of the target gene. The gene expression levels of *Sirt1* was measured to investigate whether *Sirt1* expression in the brains of different calorie-restricted mice was regulated by DNA methylation. The gene expression was measured using the RT-qPCR method. Mice of all ages and diet groups were included in this study. Following the validation of the normality, ANOVA tests were performed on gene expression data. One-way ANOVA test revealed no significant change in CR application on the mRNA expression levels of *Sirt1* in every age and dietary groups (Figure 3.9).

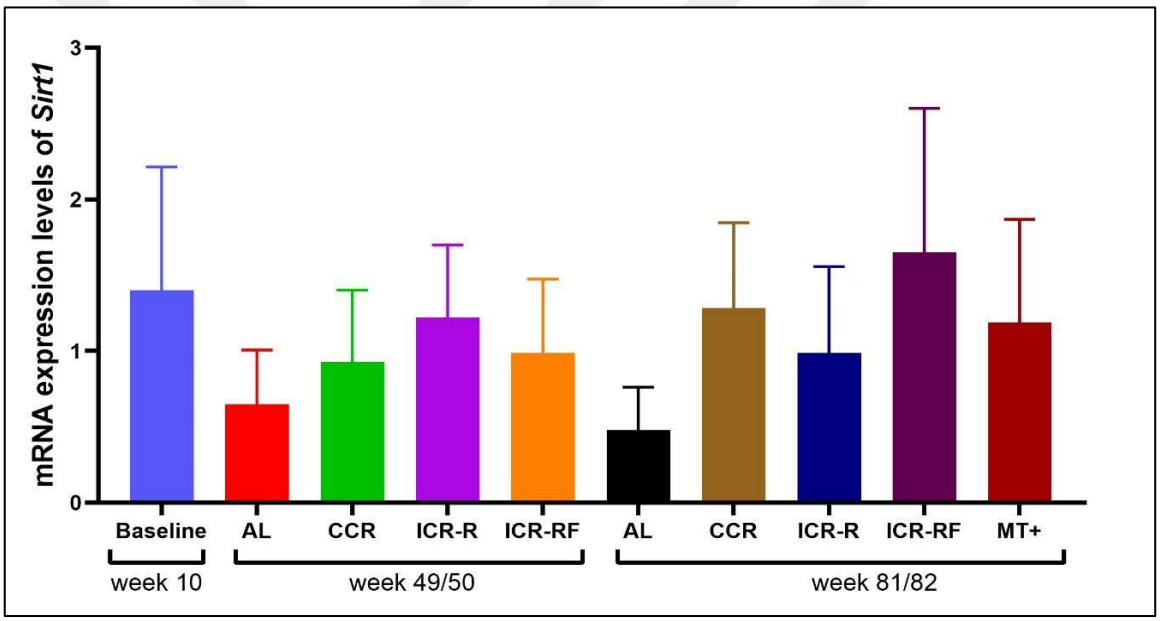


Figure 3.9. The effects of different types of CR on SIRT1 mRNA expression levels in the brain of mice at week 10, 49/50, and 81/82. The brain tissue which was taken from mammary tumour developed mice were designated as MT+. (n=4 for week 81/82 ICR-RF , n=5 for other groups)

3.3. PROTEIN EXPRESSION LEVELS IN MOUSE BRAIN

SIRT1 is an important protein involved in many cellular mechanisms. The protein level of SIRT1 was measured to understand ageing and CR-associated changes in the brain. Following the validation of the normality, ANOVAs were performed on normalised protein expression data. One-way ANOVA test revealed no significant change in CR application on

the protein expression levels of SIRT1 in every age and dietary groups. SIRT1 protein expression level was significantly lower in MT developed mice compared to the control group which is MT free and AL fed at week 81/82 when trimeric (230 kDa) of SIRT1 was analyzed (Figure 3.10 b, P=0.0201).

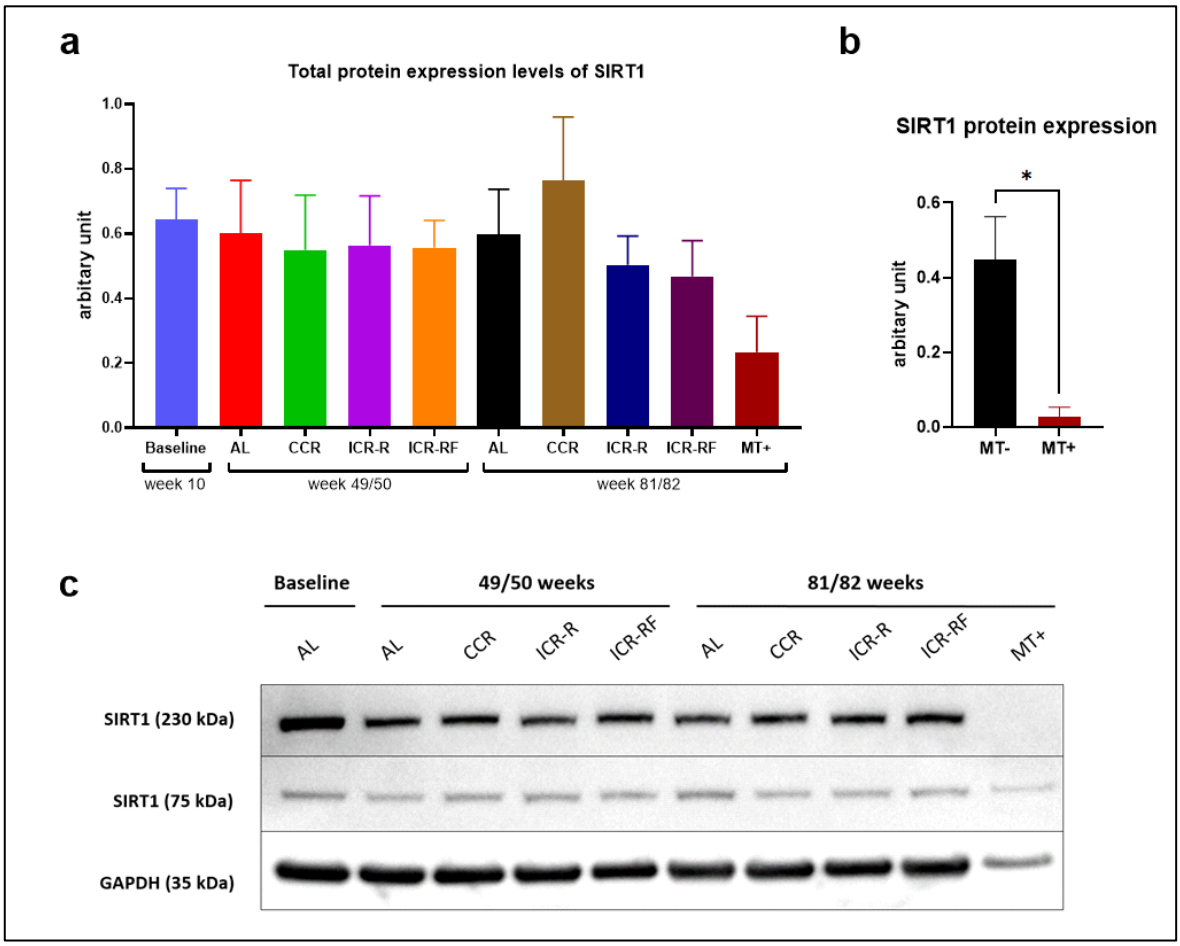


Figure 3.10. The effects of different types of CR on SIRT1 protein expression levels in the brain of mice at week 10 (baseline), 49/50, and 81/82. Bar graph for normalised band intensities of total SIRT1 (monomeric (75kDa) and trimeric (230 kDa) SIRT1) for every group (a). Bar graph for normalised band intensities of trimeric (230 kDa) SIRT1 in MT- and MT+ AL mice at week 81/82 (b). Representative immunoblot image of SIRT1 and GAPDH (loading control) (c). Each band was labelled according to the duration and dietary regimen. The brains taken from mice that developed mammary tumours were designated MT+. (n=4 for week 81/82 ICR-RF, n=5 for other groups)

3.4. CORRELATIONS

3.4.1. Correlation Between Gene Expression And DNA Methylation Levels of *Sirt1*

The correlation between mRNA expression and average DNA methylation was examined to understand whether *Sirt1* is regulated by DNA methylation. Pearson correlation revealed statistically significant difference. Average DNA methylation and *Sirt1* mRNA expression were shown to be negatively correlated (Fig.3.11 a, r= -0.3924, P=0.0053). A negative correlation between CpG specific methylation and mRNA expression of *Sirt1* was observed only in CpG1 (Fig 3.11.b, r=- 0.5698, P<0.0001) and CpG5 (Fig.3.8 f, r=-0.3792, P=0.0072).

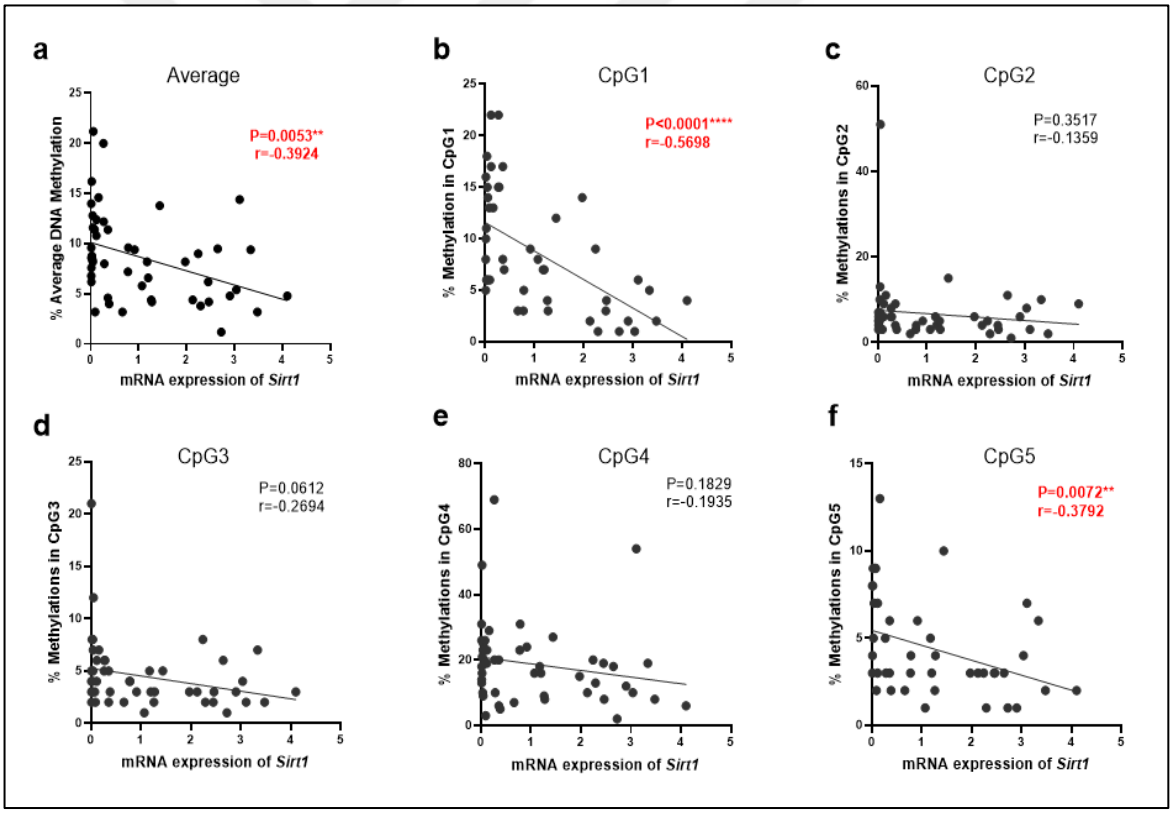


Figure 3.11. Correlations between DNA methylation in the *Sirt1* gene and *Sirt1* mRNA expression in the brain of mice at week 10 (baseline), 49/50, and 81/82. Correlation between the average DNA methylation of the 5 CpG sites in *Sirt1* and mRNA expression of *Sirt1* (a). Correlations between CpG specific DNA methylation in *Sirt1* and mRNA expression of *Sirt1* (b-f), (n=49).

3.4.2. Correlation Between Protein Expression And Gene Expression Levels of *Sirt1*

While mRNA undergoes translation, it can be controled by epigenetic mechanisms such as miRNAs. The correlation of *Sirt1* mRNA expression with the amount of protein indicates that there is no miRNA targeting *Sirt1* mRNA and inhibiting its translation. Also, post-translational modifications can affect protein levels. Pearson correlation was applied to show the correlation between the amount of mRNA and protein of SIRT1. A positive correlation between protein expression levels and mRNA expression levels of *Sirt1* is observed (Fig 3.12, r=0.3163, P=0.0268).

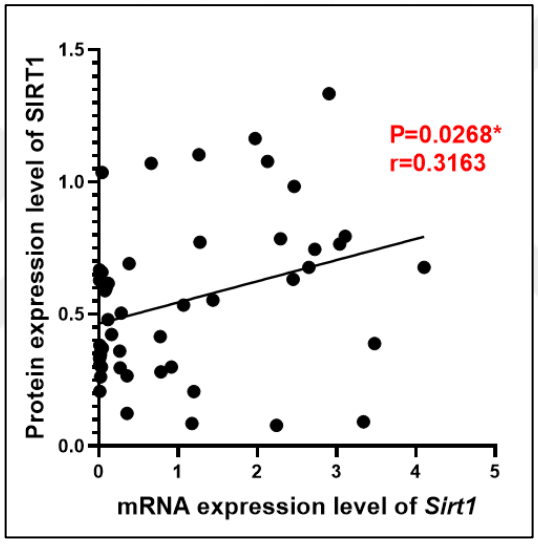


Figure 3.12. Correlations between protein expression levels mRNA expression of *Sirt1*. (n=49).

3.4.3. Correlation Between Protein Expression And DNA Methylation Levels of *Sirt1*

The correlation between protein expression and average DNA methylation was examined to understand whether *Sirt1* is regulated by DNA methylation. Pearson correlation revealed a statistically significant difference. *Sirt1*'s average DNA methylation and protein expression levels are found to be negatively correlated (Fig.3.13, r=-02926, P=0,0413).

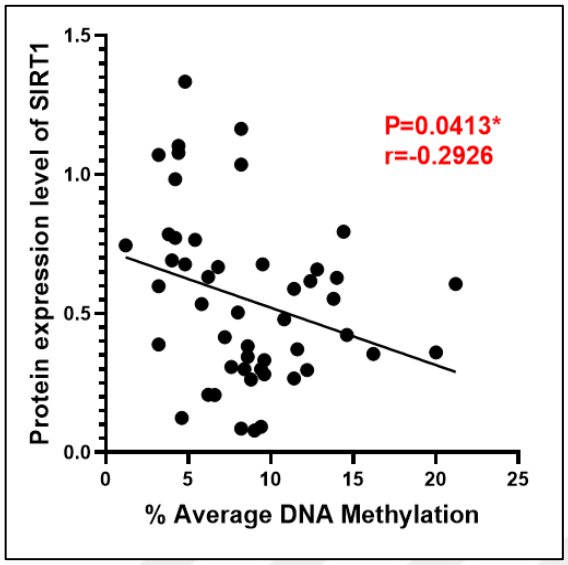


Figure 3.13. Correlations between protein expression levels and the average DNA methylation levels of *Sirt1*. (n=49)

4. DISCUSSION

Developing technologies increase the human health quality and life expectancy. With the increased in life expectancy, human being are more prone to the age related diseases. One of the main reasons for this, healthy time does not increase in direct proportion to the life expectancy. For this reason, studies to learn the cause of age-related diseases, their treatments and/or preventation are became more common in recent. CR is one of the most successful intervention approaches for reducing and reversing the consequences of ageing. Sirtuins have recently been the most studied protein family to explain the mechanisms of ageing-related diseases prognosis. But there is limited knowledge about mechanisms that regulate Sirtuins and control their expression in brain tissue.

DNA methylation has recently been discovered to be a significant, diet-induced epigenetic process regulating cellular character, also brain cell development. In the present thesis, we first sought to comprehend the role of DNA methylation on *Sirt1* expression regulation then the role of different types of CR application on *Sirt1* DNA methylation levels in the ageing mouse brain. According to our findings, we measured that the levels of *Sirt1* mRNA expression and the amount of SIRT1 protein in the brain tissue are inversely correlated with DNA methylation. We also observed neither CR application nor ageing had no significant effect on *Sirt1* DNA methylation in mouse brain.

There is much evidence on the metabolic pathways that sirtuins target and regulate, DNA methylation is inactivation mechanisms that may be used instead of genetic processes in most cases, and it normally cause transcriptional repression. DNA methylation can suppress gene transcription by inhibiting positive factors from binding to the promoter and/or attracting transcriptional co-repressors to the promoter [144]. The methylation of the first exon could also results in gene silencing [145]. When these sections of genes are methylated, the transcription machinery may be unable to reach the DNA, resulting in the gene not being transcribed [146]. Multiple studies have reported alteration in DNA methylation levels with ageing at specific CpG sites within CpG islands and in distinct brain areas, which is a major complicating factor for neurodegenerative disorders [147]. Pearson's correlation was established to check out effect of DNA methylation on *Sirt1* mRNA expression and a statistically significant difference was found (Fig 3.8, $p<0.05$). We observed that

methylation levels and mRNA expression were negatively correlated when the average of 5 CpGs was taken. We also showed that DNA methylation of each sample in CpG1 or CpG5 correlates negatively with mRNA expression of the same sample, proposing those two regions play a more important role in this regulation than other CpGs. Similar to the results we found, Simó-Mirabet et al. were able to observe a statistically significant negative correlation with mRNA expression of *sirt1* in only a few CpG regions from 22 different CpG regions they examined in the *sirt1* gene in [148]. The CpG regions examined in each study differ, and each CpG region shown to had an impact on gene expression is a candidate for therapeutic target. As expected, a positive correlation was found between protein expression and mRNA expression of SIRT1 in the mouse brain (Fig.3.9, $p>0.05$). In addition, the statistically significant negative correlation of protein expression and DNA methylation confirms the previous graphs (Fig.3.10, $p>0.05$). This result shows us that there is no mechanism that inhibits the translation of mRNA or that it is not effective enough to change the protein expression statistically.

Pyrosequencing was utilized to assess methylation of SIRT1 in sporadic early onset Alzheimer's disease (sEOAD) patient samples, but no significant differences between patients and healthy groups were found. However, areas other than promoters of other AD-related genes could be abnormally methylated in sEOAD patients, starting, or driving AD pathogenesis, implying that epigenetic modifications are a characteristic of the development of sEOAD [149] . There are no other papers that have previously measured *Sirt1* DNA methylation with pyrosequencing in the mouse brain. Although changes in *Sirt1* DNA methylation in different tissues with ageing and CR have been demonstrated, this is not sufficient to compare with our study. Epigenetic regulations differ in each tissue and cell, and the method of measurement also affects the comparison.

Both nutritional levels and CR were found to control sirtuins. SIRT1 protein expression are higher in rats fed a CR diet [46] and SIRT1 transcription is activated in a p53-dependent aspect when nutrients are depleted [150]. However, the mechanism underlying this alteration is still unknown. In age-related neurodegenerative diseases, the positive neurological benefits of CR have been well-documented [72]. This study investigated whether *Sirt1* DNA methylation changes associated with aging may be prevented by CR, supporting the idea that methylation is a possible biological modification by which CR abates brain ageing. In this study, our aim is to question the effects of different types of CR on *Sirt1* expression in the

mouse brain and the regulatory effect of DNA methylation on *Sirt1* expression. In both animal and human tissues, such as the liver, heart, kidney, brain, and lung, SIRT1 expression decreases with aging at the proteomic and transcriptomic level [151–153]. The effect of *Sirt1* on ageing is a subject that has been extensively studied recently, but the mechanism by which ageing exerts its effect on *Sirt1* expression in the mouse brain is unknown. 10, 49/50 and 81/82 weeks old mice were used in this study and the expression levels of *Sirt1* in the brains of AL fed mice was checked. At the same time, DNA methylation levels were measured. The weight gains and tumor incidences of these animals were reported in another study by our group [154]. In addition, leptin and adiponectin levels in the blood of mice were investigated and the effects of CR and ageing were observed from our group previously [22]**.** Determination of DNA methylation level of *Sirt1* has been made by pyrosequencing. Pyrosequencing results revealed no significant change between diet groups and ages as shown in Figure 3.1, 3.2 and 3.3. In addition, when the DNA methylation levels of calorie-restricted groups were compared at 49/50 and 81/82 weeks, no age-related change was observed. Regarding the impact of various CR kinds that were used for various amounts of time on the SIRT1 level, our findings demonstrated that dietary restriction, comprising all CRs and AL, did not significantly differ from one another (Fig.3.6, 3.7). Also duration has no statistically significant effect on the mRNA and protein alterations of SIRT1. In many studies, in both animal and human tissues, such as the hearth, brain, and lung, SIRT1 expression has been reported to decrease in ageing at the protein and transcription levels. [151–153]. According to numerous studies, mice' levels of SIRT1 increase in a variety of tissues in response to CR. [155–157].

The current debate over the effects of Sir2 orthologs on lifespan in nematodes and flies [156], has increased overall concern about Sir2 orthologs' function in ageing/longevity management. However it has been fiercely disputed whether sirtuins are in charge of controlling mammalian ageing and longevity because whole-body over-expression of *Sirt1* fails to allocate lifespan in mice. When they compare to wild-type mouse, these transgenic mice display antiageing traits including reduced levels of DNA damage and lower cancer frequency, greater glucose tolerance and stronger bone density [148]. Another research found that transgenic mice over-expressing SIRT1 in organs such as adipocytes and brain, but not liver or muscle, show a phenotype similar to CR [94], but SIRT1-null animals do not react to CR with a prolonged lifetime [158]. More research is needed in this area, as studies

suggest that the expression of *Sirt1* in the mouse brain varies depending on many factors and brain regions. Although *Sirt1* expression and methylation did not differ significantly from one another, the association between them may provide insight into *Sirt1* regulation.

Changes of CR on SIRT1 expression have been tested in different mammals and found that a reduced calorie diet of 40% in mice and rats and also 25% in humans is sufficient to induce an increase in SIRT1 expression [155]. The most direct link known to the association of SIRT1 with CR is SIRT1 plays role on fat accumulation in white fat cells. This may result in physiological alterations related to the SIRT1 expression levels in the cell [159]. But at the same time, SIRT1 is a histone deacetylase and plays critical job in epigenetic changes in the genome. It can cause alterations in metabolism of the proteins it targets and regulates, and it can also be regulated by epigenetic mechanisms [160,161].

Some studies examining the influence of ageing on DNA methylation levels in diverse organisms given an AL or CR diet were reported recently [116,162,163]. Although CR appeared to have no effect on DNA methylation levels in Drosophila, other investigations found that CR protects mammals from ageing-related DNA methylation alterations in various tissues [33,41,116,162–164]. Animals fed a CR diet have less alterations in genomic areas that become differentially methylated with age (aDMRs) [116,165].

Another original aspect of this study is that it found an association between breast cancer development and SIRT1 protein expression in mouse brains. As shown in Figure 3.9, SIRT1 appears as a double band in western-blot results. The upper band shows the full-length SIRT1 (230 kd) in the trimer version, while the lower band shows its monomer (75 kd). Full-length SIRT1 protein expression was statistically significantly lower in 81/82-week-old mice that developed MTs compared with AL, suggesting that MT development may be related to the amount and maintenance of SIRT1 in the mouse brain. Since the actual mechanism is not known, future studies are necessary to reveal that relationship.

SIRT1 expression was inhibited in the cerebellum and midbrain of CR mice, but induced in the cortex and hippocampus [166]. Surprisingly, mice with brain-specific over-expression of *Sirt1* have a considerable prolong in ageing and lifespan extension in both males and females, according to one study [167]. As a result, manipulating Sirtuin expression in the brain could be a promising therapeutic technique for preventing and treating age-related diseases and extending our lives. Neurodegenerative disorders such as AD and PD, are

strongly linked to increased age. The reduction in blood SIRT1 protein level was greater in patients with neurodegenerative disorders than in healthy older people, implying that decreased SIRT1 expression and activity are implicated in the progress of neurodegenerative diseases [168]. These findings point to SIRT1's decreasing expression and function as a major characteristic of the molecular pathways involved in the ageing, neurodegenerative, and metabolic disorders processes. Nonetheless, some scientific evidence suggests that the amount of SIRT1 protein and its function are not inextricably linked[169,170].

In addition, some drugs can boost SIRT1 activity in different ways, making it more effective in treating disorders. Early research has discovered that resveratrol, via the SIRT1/Sir2-dependent pathway, replicates the life-extending impact of CR in yeast and multicellular animals [171]. Resveratrol injections to mice could not just mimic CR outcomes, such as anti-ageing effect, boosting browning of white adipose tissue, and recovering disorders in mice, but can also protect against neurodegenerative diseases like Alzheimer's, ataxia, and Parkinson's disease [170–173]. In the light of all these results, the effect of DNA methylation on *Sirt1* gene regulation has emerged and this result sheds light on future studies with *Sirt1*.

5. CONCLUSION

This thesis is the first one to announce influence of different types of CR (CCR and ICR) on DNA methylation of *Sirt1* gene in ageing mouse brain. The importance of Sirtuin in lifespan and the longevity effect of CR are still controversial, despite mounting evidence suggesting Sirtuin is an attractive anti-ageing molecule implicated in enhancing health through the target molecules participating in various biological processes. SIRT1, a histone deacetylase, had been proposed as one of the essential enzymes regulating the advantageous effects of CR. While SIRT1 deletion prevents CR-mediated life-span extension, over-expression of SIRT1 mimics some physiological consequences of CR. In this study, a negative correlation between *Sirt1* expression and DNA methylation levels were determined which suggests that DNA methylation might have a regulatory effect on *Sirt1* expression. However, it was shown that CR and ageing did not make a statistically significant difference on *Sirt1* DNA methylation levels in the total mouse brain. In addition, *Sirt1* mRNA and protein expression were not altered by CR. In the present thesis, neither CR application nor ageing did not have significant effect on DNA methylation levels of *Sirt1* in brain. This may imply that CR shows its regultory effects on SIRT1 by modulating signaling molecules and mechanisms other than DNA methylation in mouse brain. For future, SIRT1 activity measurements, brain region specific analysis and DNA methylations measurements of other CpG sites in *Sirt1* will be illuminating.

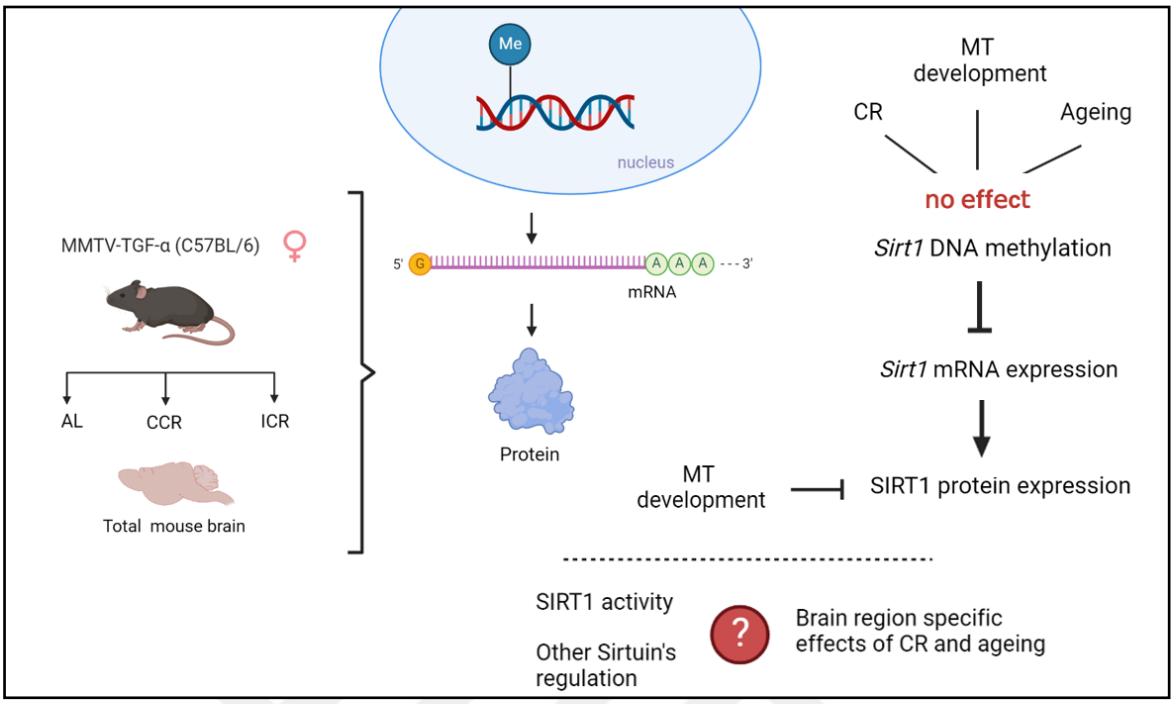


Figure 5.1. Graphical conclusion of the thesis

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