Effects of dietary polyphenols on aspects of cell molecular physiology relevant to metabolic health and ageing

Sadat Abdulla Aziz

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Institute for Cell and Molecular Biosciences

Faculty of Medical Sciences

Newcastle University

United Kingdom

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Declaration

I declare that this thesis titled "**Effects of dietary polyphenols on aspects of cell molecular physiology relevant to metabolic health and ageing"** is my own work that has not been submitted for any degree or examination in any university and that all materials used have been acknowledged and referenced.

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Abstract

Numerous studies indicate a wide range of possible health benefits from consumption of dietary polyphenols, including resveratrol and genistein. These effects include suppression of cancers, antioxidant functions and protection from diet-induced obesity.

Previous research in the laboratory using the mouse 3T3-L1 adipocyte model revealed that resveratrol affected the expression of genes involved in lipogenesis. I proposed that genistein, which has some structural similarity to resveratrol and, like resveratrol, can act through the estrogen receptor (ER), would have the same actions. However, I observed that genistein induced a change in the appearance of cells such that they resembled brown, rather than white, adipocytes. This change in appearance included the accumulation of small, multiclocular lipid droplets, typical of brown adipose tissue, which contrasted with the large lipid droplets seen white adipocytes. I thus proposed that genistein can induce the development of beige adipocytes (adipocytes with characteristics of brown adipocytes but derived from the same lineage as white adipocytes). In support of this hypothesis, genistein reduced mRNAs of genes that characterise white adipocytes (*Acaca, Fasn, Fabp4, Lipe, Rarres2, Retn*) and increased mRNAs of genes expressed specifically in brown adipocytes (*Ucp1, Tnfrsf9*) and of genes recognised as mediators of white to brown adipocyte interconversion (*Sirt1, Cebpb, Ppargc1a*) in a dose- and time-dependent manner. Measurement of mitochondrial activity revealed that basal oxygen consumption rate and mitochondrial proton leak were higher in genistein treated cells, consistent with the respiratory characteristics of beige, rather than white, adipocytes.

The presence of the ER antagonist fulvestrant reduced none of these effects of genistein on 3T3-L1 cells, indicating that this action of genistein is not through the ER. However, the responses of *Ucp1* and *Cebpb* genes to genistein were significantly attenuated by EX-527, which is a specific and potent Sirt1 inhibitor. A moderate increase (2 fold) in Sirt1 mRNA level was observed in response to treatment of 3T3-L1 cells with genistein. However, there was an increase of approximately 12 fold in Sirt1 protein, which further indicated that increased Sirt1 action is a key mediator in the change I observed. This effect of genistein on Sirt1 expression was not unique to adipocytes; a similar ER-independent effect was measured in MCF-7 breast cancer cells.

Again to develop previous work conducted in the laboratory on novel actions of resveratrol, I proposed that genistein would affect histone expression. I observed that genistein repressed transcription from a histone H3.1 (variant H3b) promoter-reporter

construct but increased the expression of histone H3 and H4 proteins. Genistein had differential actions on specific histone variant mRNAs. Notably histone H3d and H3.3 mRNAs were increased whereas all other variants measured were reduced. The ER antagonists fulvestrant and G15 did not affect any of these responses, indicating that, like the other actions studied in this body of work, these effects of genistein were though pathways independent of ERs. Observations of these ER-independent actions of genistein on histone expression extended also into 3T3-L1 cells. Gene expression through specific alterations in chromatin structure may thus be one of the mechanisms through which genistein has its reported beneficial effects.

In further work exploring ER-dependent versus ER-independent actions I determined if genistein had any effects on expression of ERs, since there is evidence that 17β-estradiol has some autoregulatory feedback action via repression of ER expression. Here I made the potentially important observation that genistein has selective actions on ERα versus ERβ, reducing mRNA corresponding to ERα while increasing ERβ mRNA. Again, these responses were not affected by ER antagonists. This action may be of benefit in the prevention or treatment of breast cancer, where ERα activity is generally detrimental while ERβ activity appears to be of benefit.

Finally, I developed the previous research on actions of resveratrol in 3T3-L1 cells by determining if the action of resveratrol to reduce mRNAs for the enzymes fatty acid synthase and acetyl Co-A carboxylase, measured previously and that I reconfirmed in the current work, was accompanied by a change in DNA methylation of the corresponding gene promoter regions, which I proposed to be a plausible mechanism of regulation given other reported effects of reseveratrol to alter DNA methylation. However, there was no effect of resveratrol on DNA methylation in the regions measured.

Together, the findings reported in this thesis indicate that a diet with a high genistein content may protect against obesity and other features of the metabolic syndrome by encouraging the development of beige, rather than white, adipose tissue. Such actions appear to be through pathways independent of the ER. Alterations in histone expression may be one of the mediating pathways, but at present I have no evidence of any causal link between the observed effects of genistein on histone expression and on adipocyte gene expression profile and phenotype. Despite excluding effects through the ER as a component of these actions of genistein, I obtained preliminary evidence that modifying the expression of ERs may be an action of genistein relevant to reported cancer-protective actions.

Abbreviations:

12-O-tetradecanoylphorbol-13-acetate……..TPA

Acetyl Co-A carboxylase……ACC

Activation function ……AF

Activator protein-1……..AP-1

Adipocyte protein 4……..AP2

AMP-activated protein kinase……AMPK

C/EBPβ response element……C/ERE

cAMP response element binding protein……CREBP

cAMP response element……..CRE

Carbohydrate responsive element binding protein……..ChREBP

CCAAT/enhancer binding.proteinβ……C/EBPβ

Centromere specific histone variant……..CENP-A

Dimethylbenz[a]anthracene ……..DMBA

DNA methyltransferase……DNMT

Endothelial nitric oxide dismutase ……..eNOD

Endothelial nitric oxide synthase ……eNOS

Epidermal growth factor receptors ……EGFRs

Estrogen receptor-α isoform 39 ……..ER-α 39

Estrogen receptor-α isoform 46 ……ER-α 46

Estrogen receptor-α isoform 66 ……ER-α 66

Estrogen receptor-α……..ERα

Estrogen receptor-β……..ERβ

Estrogen response element……ERE

Eukaryotic translation initiation factor-4γ……EIF4G

Fat specific protein 27……..FSP27

Fatty acid binding protein 4…….. Fabp4

Fatty acid synthase……..FAS

Flavin adenine dinucleotide……FAD

Forkhead box proteins ……FOXO

General control non-repressed protein-5……..GCN5

Glucose Transporter 1……GLUT1

Glucose Transporter 4 ……GLUT4

Glutathione peroxidase ……GPX

G-protein-coupled receptor-30……GPR30

Histone H3 lysine 27 dimethylation……H3K27m2

Histone H3 lysine 9 dimethylation……H3K9m2

Histone H3 lysine 9 trimethylation……H3K9m3

Histone H3 lysine 9 trimethylation……H3K9m3

Histone H3 lysine 56 acetylation……H3K56ac

Histone H4 lysine 16 acetylation……H4K16ac

Hormone sensitive lipase……HSL

Human multipotent adipose derived stem cells ……hMADS

Human Simpson-Golabi-Behmel syndrome preadipocytes ……SGBS

Human telomerase reverse transcriptase ……hTERT

Human telomerase reverse transcriptase ……hTERT

Human umbilical vein endothelial cells ……HUVEC

Interferon ……IFN

Interleukin……IL

Liver X receptor α ……LXRα

Lysine……K

Matrix metalloproteinase 3……MMP3

Matrix metalloproteinase-9 ……MMP9

Metallothionein……MT

MicroRNA ……miRNA

Mitochondrial transcription factor……TFAM

Mitogen activated protein kinase……MAPK

Mouse embryonic fibroblast ……MEFs

Nicotinamide adenine dinucleotide……NAD

Nitric oxide……NO

Non-esterified fatty acids……NEFAs

Non-POU-domain-containing octamer binding protein…… NONO

Normal murine mammary epithelial cell line……..HC11

Nuclear receptor co-repressor……NCoR

Nuclear respiratory factor 1 and 2 ……NRF1 and 2

Peroxisome proliferative activated receptor gamma coactivator 1 alpha……PGC1α

Peroxisome proliferator activated receptor response element……PPRE

Peroxisome proliferator-activated receptor-α……PPARα

Peroxisome proliferator-activated receptor-γ ……PPARγ

Phosphatase and tension homolog……PTEN

Positive regulatory domain contain 16……PRDM16

Positron emission tomography ……PET

Post translational modifications ……PTM

Proliferative cell nuclear antigen……PCNA

Protein kinase A……PKA

Quinone oxidoreductase 1 ……NQO1

RAS associated domain family-1α ……RASSF-1α

Retinoic acid response element……RARE

Silencing mediators of retinoid and thyroid hormone receptors……SMRT

Simian Virus-40 T-antigen……SV-40 Tag

Single nucleotide polymorphism……SNP

Site-1 protease……S1P

Small interfering RNA……siRNA

Sodium/ glucose co-transporter-1……SGLT1

Specificity protein-1……SP-1

Stem loop binding protein ……SLBP

Stem loop binding protein interacting proteins 1……SLIP1

Steroid receptor coactivator-1 ……SRC1

Sterol regulatory element-binding protein-1……SREBP-1

Sulfotransferase ……SULTs

Superoxide dismutase ……SOD

Telomerase activity ……TLMA

Thyroid receptor β……TRβ

Thyroid response element……..TRE

Topoisomerase 1……Topo1

Tumor necrosis factor receptor superfamily, member 9 ……Tnfrsf9

Tumor necrosis factor……..TNF

Type 2 deiodinase……..D2

U7 small nuclear ribonucleic proteins……U7 snRNP

UDP-glucurunosyltransferase ……..UGTs

Uncoupling protein 1……..UCP1

Vascular endothelial growth factor ……VEGF

Vascular endothelial growth factor receptors ……..VEGFRs

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Genistein promotes a gene expression profile characteristic of brown rather than white adipocytes and increases Sirt1 expression in mouse 3T3-L1 cells (Oral)
Experimental Biology 2014, Convention centre, San Diego, California, 29.04. 2014.

Induction by genistein of a gene expression profile in mouse 3T3-L1 cells indicating conversion of white to beige adipocytes (Oral)
Diet, gene regulation and metabolic disease conference, Robert Gorden University, Aberdeen, 25.03.2015.

Genistein promotes a gene expression profile characteristic of brown or beige, rather than white, adipocytes and increases Sirt1 expression in mouse 3T3-L1 cells (poster)
Obesity: A Physiological Perspective, Newcastle United Football Club, Newcastle upon Tyne Newcastle, United Kingdom, 10-12.09.2014.

The dietary soya component genistein promotes conversion of cultured white fat cells to brown fat cells (Oral presentation)
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Genistein promotes a gene expression profile characteristic of brown rather than white adipocytes and increases Sirt1 expression in mouse 3T3-L1 cells (oral and poster presentation)
HNRC anniversary symposium, Lindisfarne Room, Kings Road, Newcastle University, 15.10.2014.

The dietary isoflavone genistein promotes features of brown over white adipocytes in a cell line model (Oral)
HNRC anniversary symposium, Lindisfarne Room, Kings Road, Newcastle University 29/11/2012

Chapter 1

1. Introduction

1.1. Dietary polyphenols

Natural polyphenols occur most abundantly as water-soluble secondary metabolites of plants (Ho, 1992) and are primarily produced by plants in response to external stress stimuli such as infection and UV irradiation. Their primary functions are to protect plants against fungal and bacterial infection, and they might act as signals that regulate processes of symbiosis (Mandal et al., 2010).

Structurally, polyphenols contain an aromatic ring carrying more than one (phenol) hydroxyl substituent. Some polyphenols including isoflavones, stilbenes and lignans, structurally and possibly functionally resemble 17β-estradiol, which is the mammalian endogenous estrogen hormone. These compounds may thus have some effects through activating estrogen receptors (Daayf, 2008) or their action is likely to be through other pathways.

In recent years, there has been a worldwide trend towards promoting the consumption of some dietary polyphenols to derive potential health beneficial effects. The dietary polyphenols resveratrol and genistein are the main focus of the current work. The particular actions studied relate to their possible use as agents to reduce lipogenesis, and thus to alleviate obesity-related health problems such as cardiovascular disease, type-2 diabetes and non-alcoholic fatty liver disease.

1.1.1. Classification of polyphenols

The structural and chemical diversity of plant polyphenols have led to the use of different systems of classification. However, this group of compounds are generally classified as flavonoids, phenolic acids, polyphenolic amides and non-flavonoid polyphenols. Over 4000 flavonoids have been recognised and are characterised by having two phenolic rings as well as one heterocyclic ring. Isoflavones, one of the subgroups of the flavonoids, are characterized by the second phenolic ring binding to the C3 of the heterocyclic ring. The isoflavones most commonly consumed in human diets are genistein, diadzein and glycitein. They occur in general in the greatest abundance in the leguminous plant family which includes peas and beans (Tsao, 2010).

The stilbenes are a separate group of dietary polyphenols belonging to the non-flavonoid division and characterised by having two phenolic rings connecting via a methylene bridge. In the context of the human diet resveratrol is the representative of this group that has been studied most extensively (Soleas et al., 1997; Tsao, 2010; Yang and Xiao, 2013)

1.1.2. Genistein

Like other dietary polyphenols, notably diadzein and glycitein, genistein is abundant in peas and beans (Tsao, 2010). Because of its structural similarities with estrogen, it is also described as a non-steroidal phytoestrogen (Manach et al., 2004). Genistein is one of the active components of several traditional medicinal plants, particularly plants used in traditional Chinese medicine, and it has been used for centuries to treat several health disorders (Lee et al., 2004; Sham et al., 2014). A wide range of possible health benefits related to consuming genistein have been indicated in numerous in vitro and in vivo studies. These possible positive actions include anti-cancer activity (Wang et al., 2002), antioxidant effects (Sales and Resurreccion, 2013), and antilipogenic activity (Kim et al., 2010). Several pathways and mechanisms through which genistein might regulate cellular activities at the molecular level have been identified and include estrogenic effects (Magee and Rowland, 2004), tyrosine kinase inhibition (Kasai and Kikuchi, 2010) and epigenetic effects (Fang et al., 2005; Dolinoy et al., 2006). To elucidate further how genistein can influence cell and tissue biology, thereby the implications for human health of consuming genistein, further work is required.

1.1.2.1. Chemical structure of genistein

The attachment of the second phenolic ring of genistein to C3 of the heterocyclic ring and the relatively similar pattern of distribution of the hydroxyl groups lend a structural and probably functional similarity between genistein and 17β-estradiol (Ogbuewu et al., 2010). The glycosylated form of genistein (glycone) is known as genistin and it carries the sugar moiety on C7 of the first phenolic ring of the parental genistein backbone (Coward et al., 1993). The structure of genistein, genistin and 17β-estradiol are shown in Figure 1.

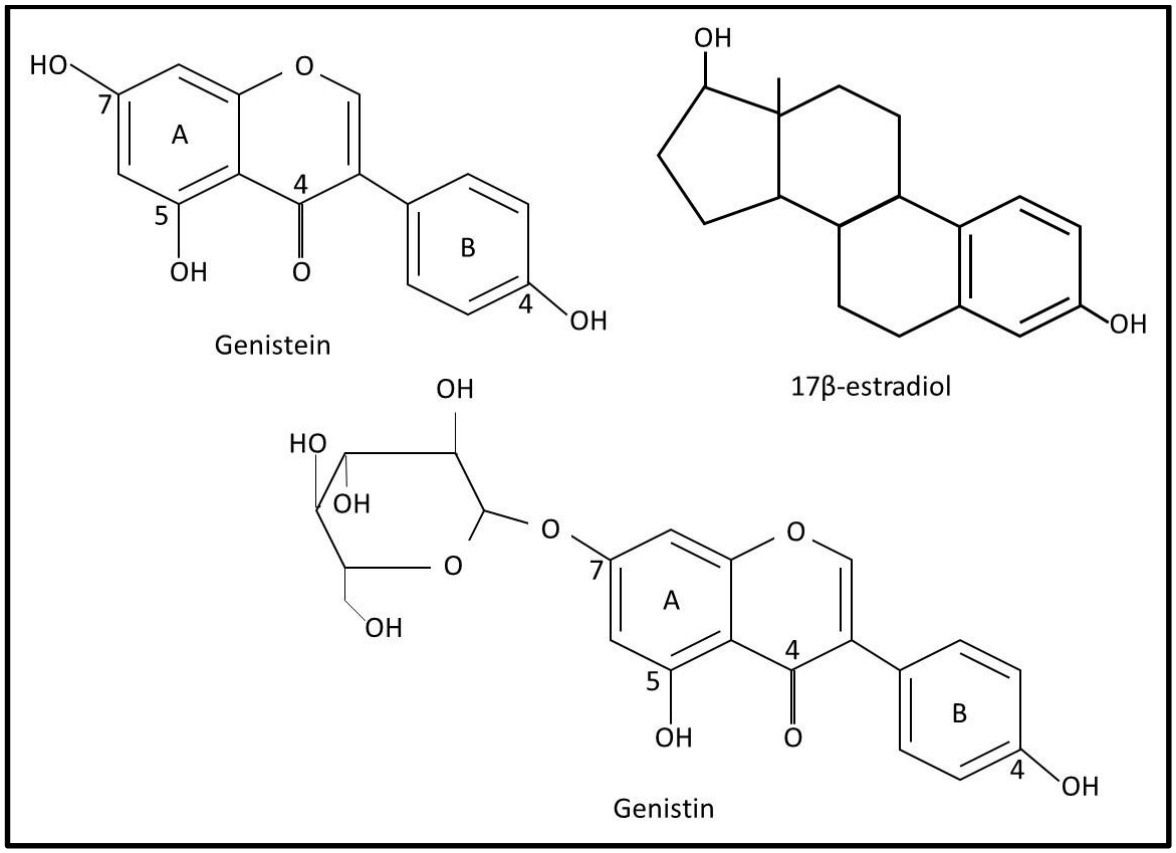


Figure 1. 1: Chemical structures of genistein, genistin and 17β-estradiol

1.1.2.2. Sources of genistein

Genistein is particularly abundant in soya and its related products such as soya milk, soya sauce and tofu (Horn-Ross et al., 2000; Yamamoto et al., 2001; Otaki et al., 2009). Genistein is present in these types of food in different forms including genistin (genistein glycone) and genistein aglycone (Messina et al., 2006). The process of fermentation used to produce products such as tofu dramatically increases the ratio of aglycone to genistin. The concentration of isoflavones (mainly genistein, daidzein and glycitein) in the soyabean plant is highly variable and it depends on environmental factors such as temperature (Zhang et al., 2002) and microbial infection (Formela et al., 2014). In general, the isoflavone concentration of fresh soyabeans ranges between 600 and 4000 mg/kg while the isoflavone content of soymilk is estimated to be between 30 and 170 mg/L (Manach et al., 2004). In comparison to other isoflavones in these food products, the concentration of genistein is particularly high and has been estimated to comprise approximately 50% (Delmonte et al., 2006). Trace of genistein are found in other types of food including wheat bran, bread (white or whole grain), oatmeal, rice (white or brown,

boiled) and some vegetables, for example, raw tomato, watercress, okra, mushroom, cucumber, sprouting broccoli and aubergine (Liggins et al., 2000).

1.1.2.3. Daily intake of genistein

Based on epidemiological and experimental studies, it has been proposed that a daily intake of at least 60 mg of isoflavones (Valtueña et al., 2003) or consumption of around 25 g of soya protein per day is required to drive health beneficial effects of genistein and to keep the concentration in blood within a physiologically active level. In some Asian countries, particularly Japan and China, where there is a low incidence of cancer and other chronic diseases, the amount of dietary isoflavone consumption is typically high and it has been estimated to be around 20-50 mg per day, with about 10% of the Asian population consuming around 100 mg of isoflavones per day, with older Japanese people having even higher intakes (Messina et al., 2006). In contrast, in some European countries including the UK, and also in the United States, daily dietary isoflavone intake is typically low, and has been estimated to be around 1 mg per day (Marie-Agnes et al., 2003; Chun et al., 2007), This low level of isoflavone intake may be insufficient for biological activity (Horn-Ross et al., 2002; Valtueña et al., 2003).

1.1.2.4. Genistein absorption, metabolism and excretion

Genistein aglycone is absorbed mainly within the small intestine by passive diffusion. The glycosylated form of genistein (genistin) is less readily absorbed (Messina et al., 2006). Genistin is deglycosylated by both bacterial and intestinal β-glucosidase enzymes, and this processing is to release genistein aglycone comprises the primary route for absorption (Setchell et al., 2002; Cederroth et al., 2012). After absorption, it appears that genistein is largely conjugated with glucuronic acid (Setchell, 2000; Chen et al., 2005; Zhou et al., 2008) or sulfate, particularly within the liver and enterocytes (Ronis et al., 2006), through the action of UDP-glucurunosyltransferases (UGTs) and sulfotransfrases (SULTs) (Williamson and Manach, 2005). These post-absorptional modifications of genistein increase solubility and may be important for transport by the blood as well as for renal excretion. Genistein has a high affinity for serum albumin, which thus may be an important carrier for cell and tissue delivery (Cao *et al.*, 2013a). In comparison with other isoflavones including diadzein, genistein has a slightly longer half-life (8.5 h) within the blood (Izumi et al., 2000; Cohen et al., 2011). The longer half-life is likely to be relevant to

bioavailability of the tissues and cells. Genistein is excreted predominantly through the urinary system and biliary tract (Seow et al., 1998; Watanabe et al., 1998; Zhou et al., 2008). Most genistein excreted into the intestine via bile can be re-absorbed through enterohepatic circulation, particularly after their de-conjugation by glucuronidase enzymes within the intestine (Sfakianos et al., 1997; Zhou et al., 2008). Therefore, the entero-hepatic circulation is also considered important for genistein bioavailability (Wang et al., 2006). The process of absorption of genistein is shown in Figure 2.

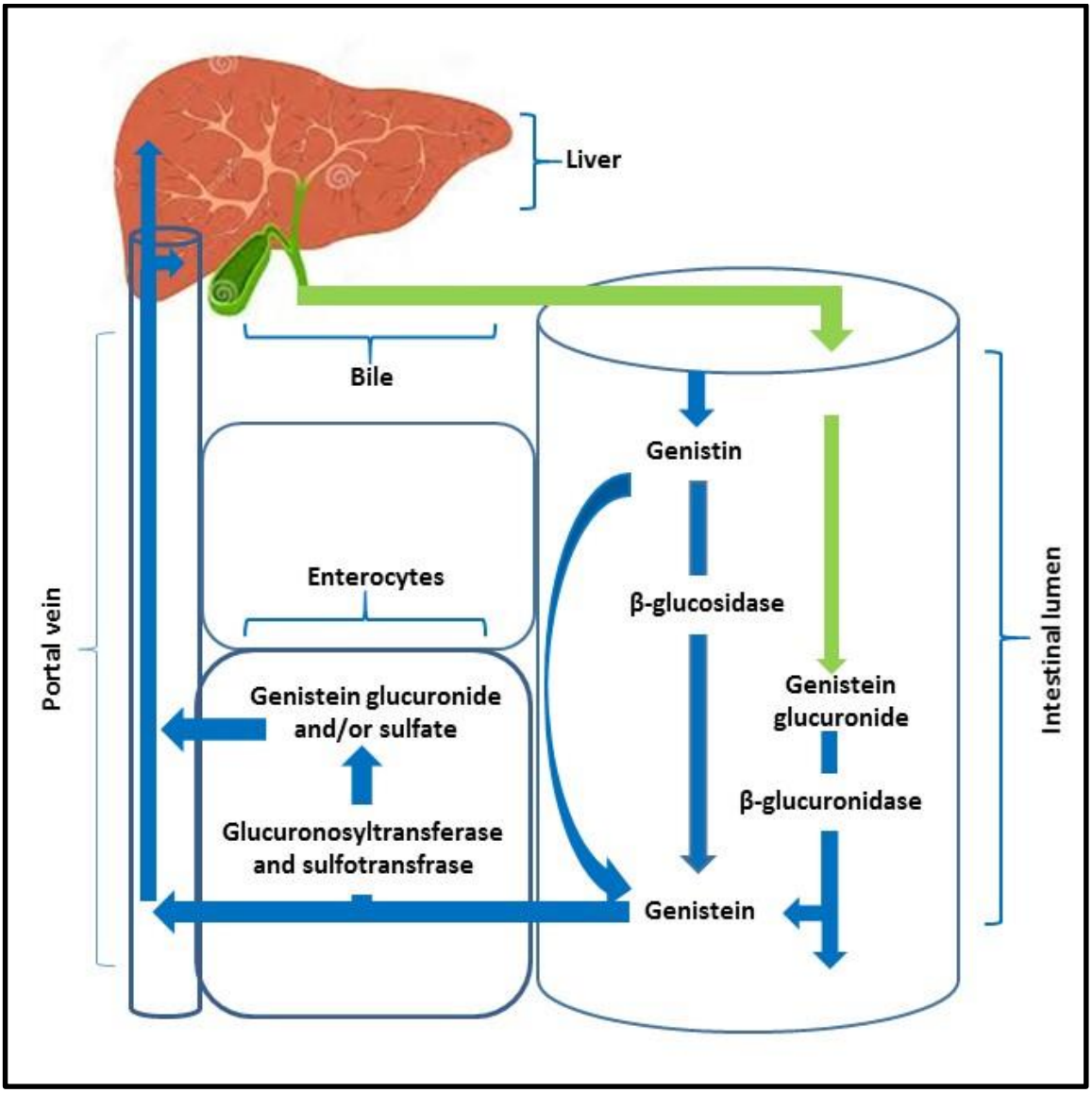


Figure 1. 2: Genistein absorption, metabolism and entero-hepatic circulation. After ingestion, β-glucosidase enzymes catalyse the conversion of genistin into genistein. Genistein is passively absorbed by the intestine. The absorbed genistein either enters into the circulation unchanged or after its conversion into genistein glucuronide and sulfate by glucurunosyltransferase or sulfotransferase within the enterocytes. The genistein glucuronide excreted into the intestine via bile is reabsorbed by the intestine after its deconjugation by β-glucuronidase within the intestine.

1.1.2.5. Genistein bioavailability

Under physiological conditions genistein bioavailability is highly variable and depends on the type of food matrix (liquid or solid), individual and cultural habits, chemical form of the isoflavone (glycosylated or not) (Jefferson and Williams, 2011) and even perhaps sex as its bioavailability appears to be higher in females than in males (Cassidy et al., 2006). Fermented dietary isoflavone sources generally have higher bioavailability than non-fermented sources because of the conversion of the glycosylated form to aglycone by the microbial β-glycosidase enzymes involved in processing, thus releasing a moiety available for direct passive absorption by intestinal cells (Chen et al., 2012). Nanoparticle technology offers some promise to elevate the bioavailability of pharmacological agents, including genistein (Tang *et al.*, 2011; Ha *et al.*, 2013).

The highest physiological plasma concentrations of genistein (5 μM) have been recorded among the Japanese. This is likely to be related to cultural eating habits (Banerjee et al., 2008). High concentrations of genistein (2.53 μM) have also been measured in the blood of infants feeding soya milk (Setchell et al., 1997). Interestingly, very high plasma concentrations (4-27.1 μM) of genistein were measured in prostate cancer patients after oral administration of two different doses of genistein (300 mg/day for 28 d then 600 mg/ day for another 56 d) (Miltyk et al., 2003). Genistein is not distributed uniformly between the body tissues. A different genistein concentration was measured in different tissues under conditions in which the concentration of genistein in the blood was 6-8 μM, for example, liver 7.3 pmol/mg, mammary gland 2.5 pmol/mg, ovary 1 pmol/mg, thyroid 1.18 pmol/mg, brain 0.04 pmol/mg (Chang et al., 2000). Variations in genistein concentration between different tissues may be a reason underlying tissue-dependent physiological and pharmacological effects of genistein.

1.1.2.6. Health benefits of genistein

Benefits to health attributed to consumption of genistein in the diet include cancer suppression, antioxidant effects and anti-lipogenic action. The following sections provide some pieces of evidence in support of this claim.

1.1.2.6.1. Anticancer effects of genistein

Cancer is characterised by uncontrolled cell division as a result of a genetic defect affecting proto-oncogenes and tumor-suppressor genes followed by local and systemic spread. The process of cancer development (carcinogenesis) can be divided into several stages including initiation, promotion, progression and invasion (Lodish *et al.*, 2004). A considerable body of literature focuses on anti-cancer effects of genistein. Epidemiological, clinical and experimental studies support the view that genistein can have impact on different stages of the process of carcinogenesis including initiation, promotion, propagation and invasion, and may also selectively induce cancer cell damage and apoptosis (Kim et al., 2014).

Epidemiological studies have revealed that the incidence of cancer among Japanese and Chinese people is particularly low compared to other populations. As already noted, this might be related to a substantially higher intake of dietary isoflavones (Nagata, 2000; Yamamoto et al., 2003; Kurahashi et al., 2007; Lampe et al., 2007; Banerjee et al., 2008; Iwasaki et al., 2008; Lee et al., 2009). Other supportive evidence includes the finding that dietary supplementation of genistein has reduced risk of cancer in a chemically induced prostate cancer in rat and in a transgenic mouse model of prostate cancer (Mentor-Marcel et al., 2001; Wang et al., 2002; Mentor-Marcel et al., 2005). Similarly, genistein has been found to impair properties related to cancer progression and proliferation in different cell models including breast cancer cells (MCF-7) (Fan et al., 2013), human prostate cancer cells (PC3 and DU145) (Chiyomaru et al., 2012), ovarian cancer cells (Gercel-Taylor et al., 2004), and human leukemic mast cells (Alexandrakis et al., 2003).

One beneficial model of action of genistein is likely to be impairment of cancer cell survival and induction of cell cycle arrest (Hewitt and Singletary, 2003) and apoptosis (Thasni et al., 2008). The induction of cell cycle arrest and apoptosis in cancer cells by genistein appears to be related to the activation of the P53 pathway in response to DNA

damage and hyperproliferation. Pronounced effects of genistein on P53 signalling were reported in colon cancer cell models (HCT-116 and SW-480) (Zhang et al., 2013b). In addition, genistein appears to down-regulate the expression of some genes involved in cancer invasion and metastasis including matrix metalloproteinase-3 (MMP3) and matrix metalloproteinase-2 (Yu et al., 2012; Betancourt et al., 2014), These zinc-dependent enzymes are responsible for tissue matrix as well as non-matrix protein degradation (Duffy et al., 2000; Nagase et al., 2006) and are typically expressed at high levels in highly invasive cancers (Balduyck et al., 2000).

Genistein may also decrease the risk of cancer development through epigenetic modulation of factors which are important in the process of carcinogenesis, including human telomerase reverse transcriptase (hTERT). Genistein (100 μM) treatment was found to decrease hTERT expression in both benign and malignant breast cancer cell lines (MCT10AT and MCF-7), an effect that seems to be mediated via remodelling the chromatin structure by increasing H3K9 trimethylation and decreasing H3K4 dimethylation, which are markers of gene repression (Li et al., 2009). Moreover, genistein was found to decrease the DNA methylation status of some tumor suppressor genes including phosphatase and tension homolog (PTEN) via reducing the expression and activity of the DNMT enzymes (Xie et al., 2014).

1.1.2.6.2. Antioxidant effects of genistein

Reactive oxygen species, particularly imbalanced oxidative stress, are mediators of molecular and structural damage of cellular components including DNA and other cellular organelles, and are widely considered to be a principle cause of cellular and systemic disturbances including life threating diseases such as vascular damage, neurodegenerative disease and cancer (Sales and Resurreccion, 2013). Any agent with the ability to decrease or prevent oxidative damage could potentially be used to reduce these effects of free radicals. Genistein is one of the dietary agents with possible potent anti-oxidant properties. Over the past few decades, several studies have revealed that genistein, even at physiological levels (0.1 to 5 μM), can protect human lymphocytes and human leukaemia T-cells from iron-induced oxidative damage (Foti et al., 2005). Likewise, genistein can reduce oxidative stress-associated cardiovascular disorders (Arai et al.,

2000; Nagata, 2000) and protect DNA in human skin fibroblasts from damage by UV light (Iovine et al., 2011).

It has commonly been assumed that genistein has direct antioxidant activities because of its ability to donate protons from its hydroxyl groups to oxidized substrates within the cell (Pietta, 2000; Rimbach et al., 2003; Rimbach et al., 2004; Kruk et al., 2005). However, genistein can also reduce oxidative stress indirectly by enhancing the expression and activity of antioxidant enzymes. The expression and/or the activities of several enzymes including superoxide dismutase (SOD) (Ho et al., 2003; Borra´s et al., 2006; Park et al., 2010) catalase (Park et al., 2010; Georgetti et al., 2013), glutathione peroxidase (GPX) (Hernandez-Montes et al., 2006; Georgetti et al., 2013) and quinone oxidoreductase 1 (NQO1) (Wiegand et al., 2009), were seen to be increased by genistein in both in vivo and in vitro studies.

Genistein may also improve cellular defence against oxidative stress via stimulating the expression of metallothionein (MT). it should be noted that MT is a protein that chelates metal ions and is responsible for the storage of heavy metals within the cell and thus protection from the hazardous effects of metal ion-related free-radical generation and toxicity (Perron and Brumaghim, 2009).

In contrast, however, there are some concerns that dietary flavonoids including genistein may under some conditions have pro-oxidant effects (Halliwell, 2008). For example, a high dose of genistein (200 μM) was found to induce reactive oxygen species formation in primary pig muscle cells (Chen *et al.*, 2014).

1.1.2.6.3. Other effects of genistein

In addition to anti-cancer and anti-oxidant activities, genistein has been reported to have several other health beneficial effects. For instance, multiple lines of evidence support the view that genistein can increase bone density and osteogenesis, especially in individuals who are at high-risk of osteoporosis including postmenopausal women and elderly people (Setchell and Lydeking-Olsen, 2003). Additionally, a cohort study showed that the incidence of bone fracture was low among Chinese postmenopausal women who consumed more than 38 mg of soya isoflavone per a day (Zhang et al., 2005). Another likely beneficial effect of genistein relates to inhibition of angiogenesis, particularly in cancer, via decreasing expression of vascular endothelial growth factor receptors(VEGFR)

and epidermal growth factor receptors (EGFR) (Wang et al., 2011; Yu et al., 2012). Genistein may also act as an antihypertensive agent by improving the vascular endothelial nitric oxide (NO) level as a consequence of upregulating the expression level of endothelial nitric oxide synthase (eNOS), which is involved in the synthesis of nitric oxide (Si and Liu, 2008). Genistein can also have an anti-diabetic action. Dietary genistein (0.02% w/w) supplementation in the C57BL/KsJ-bd/bd mouse, which is a model of type 2 diabetes, was found to result in an improved metabolic profile including lower blood glucose, improved glucose tolerance, and reduced plasma total cholesterol, triglyceride and free fatty acids (Ae Park et al., 2006).

1.1.2.7. Adverse effects of genistein

There are controversial reports of possible adverse effects of genistein, especially on the reproductive system. High consumption of genistein could possibly be associated with infertility and reproductive disorders as a consequence of structural and functional similarity with 17β-estradiol. In female mice, for example, genistein was found to increase uterine weight and led to follicular ovarys and irregular estrous cycle though without any effects on fertility (Cimafranca et al., 2010). However, perinatal exposure of female rats with genistein led to fertility abnormalities (Nagaoa et al., 2001). There are also conflicting data about effects of genistein on the male reproductive system. For example, antenatal and postnatal genistein exposure in rodents had no adverse effects on the male reproductive system including sperm quality and testicular size (Nagaoa et al., 2001; Fieldena et al., 2003). In contrast, other studies found that genistein consumption in the same species was associated with a reduction in testicular size, spermatogenesis and urogenital abnormalities (Atanassova et al., 2000; Wisniewski et al., 2003).

With respect to actions on other systems, there is a large body of conflicting data concerning genistein toxicity, and results from in-vivo compared with in-vitro studies seem particularly discordant. In vitro, it was found that genistein had genotoxic effects and promoted micronucleus formation in several cell lines including Chinese hamster V79 cells, human lymphoblastoid cells, and human blood lymphocytes (Morris et al., 1998; Kulling et al., 1999; Virgilio et al., 2004). Conversely, in female Wistar rats genistein had potent antigenotoxic effects against a polycyclic aromatic hydrocarbon, dimethylbenz[a]anthracene (DMBA), which included impeding micronucleus formation

and preventing chromosome aberrations (Pugalendhi et al., 2009). Also it was demonstrated that genistein had no mutagenic or clastogenic effects in mice and rats when treated with 20 mg or 2000 mg genistein per kilogram body weight for 14 days (McClain et al., 2006). Similarly, in humans oral administration of 300 mg to 600 mg of genistein per day for 84 days had no measured genotoxic effects (Miltyk et al., 2003), and it was concluded that subchronic and chronic exposure to genistein (50, 150, 500 mg/kg per day for 4-52 weeks) did not result in any systemic toxicity in dogs (McClain et al., 2005).

1.1.2.8. Proposed mechanisms of action of genistein

Genistein appears to have pleiotropic cellular actions thus it is difficult to attribute physiological effects to specific actions at the molecular level. However, there is good evidence that genistein can act as an estrogen receptor (ER) ligand. Lower concentrations of genistein (up to 3.7 μM) appear to have estrogen-like effects (Chen and Donovan, 2004; Magee and Rowland, 2004) in different cell models including MCF-7 (breast cancer) cell, HeLa (human cervical epithelial cells), Caco-2 (human intestinal) cell, KS483 (murine preosteoblastic) cells and mouse bone marrow cells (Maggiolini et al., 2001; Dang et al., 2003; Chen and Donovan, 2004). Effects of genistein mediated through its action as an ER ligand depends on the ERα/ERβ ratio. In cells with a high ERα/ERβ ratio genistein appears to stimulate proliferation and inhibit apoptosis, while in cells where this ratio is low genistein seems to induce cell cycle arrest and stimulate apoptosis (Pons et al., 2014). The action of genistein at higher concentrations appears to be via other cellular pathways (Dang et al., 2003). Actions measured at higher concentrations include inhibition of tyrosine kinase and topoisomerase activities (Salti et al., 2000; Chen and Donovan, 2004; Kasai and Kikuchi, 2010). Additionally, genistein was found to reduce expression of topoisomerase II in HeLa cells (Zhou et al., 2009). Genistein could thus induce cell cycle arrest and promote apoptosis via inhibition of tyrosine kinase and/or topoisomerase (Schmidt et al., 2008). Likewise, this function of genistein can be maintained through decreasing the expression of cell cycle regulatory factors, including cyclin D, and increasing accumulation of cyclin B (P62) protein at G2/M, which is an important indicator of cell cycle arrest (Cappelletti et al., 2000; Hewitt and Singletary, 2003; Chen and Donovan, 2004).

Genistein may also act as a ligand for transcription factors including PPARγ (Dang et al., 2003) and PPARα (Kim et al., 2004). Genistein also can directly inhibit the function of GLUT4 and GLUT1 transporters, which are involved in the transport of dehydroascorbic acid, glucose, methylglucose and deoxyglucose across the cellular membranes (Vera *et al.*, 1996; Bazuine *et al.*, 2005; Chou *et al.*, 2010).

Genistein has additionally been shown to have epigenetic actions including effects on DNA methylation and histone modification. Some of these actions may be through decreasing the expression of DNMT enzymes; genistein (50-100 μM) decreased DNMT expression and activity in a dose and time dependent manner in MCF10AT, MCF-7 and MDA-MB-213 cells (Li et al., 2009; Zhang et al., 2013a; Xie et al., 2014). Further, genistein was also found to increase the expression level of histone deacetylase enzymes (Zhang et al., 2013a).

Additionally, genistein can act as a potent antioxidant, as discussed in section 1.1.2.6.2, providing another possible mode of action through which genistein could prevent cellular and DNA damage. In summary, many molecular actions of genistein have been demonstrated most of which actions may underline some of the physiological manifestations of genistein consumption. However, further research in this area is merited to attribute unequivocally specific beneficial outcomes to particular molecular actions.

1.1.3. Resveratrol

Like genistein, resveratrol is a dietary polyphenol. However, resveratrol (3,5,4 trihydroxystilbene) belongs to the stilbene group (Tsao, 2010; Yang and Xiao, 2013). Again, similar to genistein, resveratrol has been described as a phytoestrogen because of its structural similarities with 17β-estradiol. It appears that resveratrol can act on the ER as either an agonist and/or antagonist (Bhat et al., 2001). Observed biological effects of resveratrol include epigenetic actions some of which are likely to be attributable to altering the S-adenosylmethionine to S-adenosylhomocysteine ratio or activating enzymes that catalyse DNA methylation (DNMTs) and histone modification (Sirt1) (Park *et al.*, 2012a). Resveratrol also has been found to affect the expression of genes involved in the modification of lifespan including Sirt-1, Sirt-3, Sirt-4 and Forkhead box O genes (FOXO1, FOXO3a) (Das et al., 2011). Other observed actions to reduce features associated with aging include improved aortic elasticity and reduced inflammation and vascular endothelial damage (Pearson et al., 2008).

1.1.3.1. Structure of resveratrol

Resveratrol exists as cis and trans-resveratrol isomers (Anisimova et al., 2011). Trans-resveratrol is sensitive to UV light irradiation, which causes its conversion to cis-resveratrol (Frémont, 2000). Most studies of biological action have been on tans-resveratrol, which is biologically active whose form is most easily available commercially (Pervaiz, 2003). Plant tissues contain resveratrol in glycosylated and methylated forms in addition to the parent compound (Bavaresco et al., 2002; Regev-Shoshani et al., 2003; Moreno-Labanda et al., 2004). The chemical structures of resveratrol are shown in Figure 3.

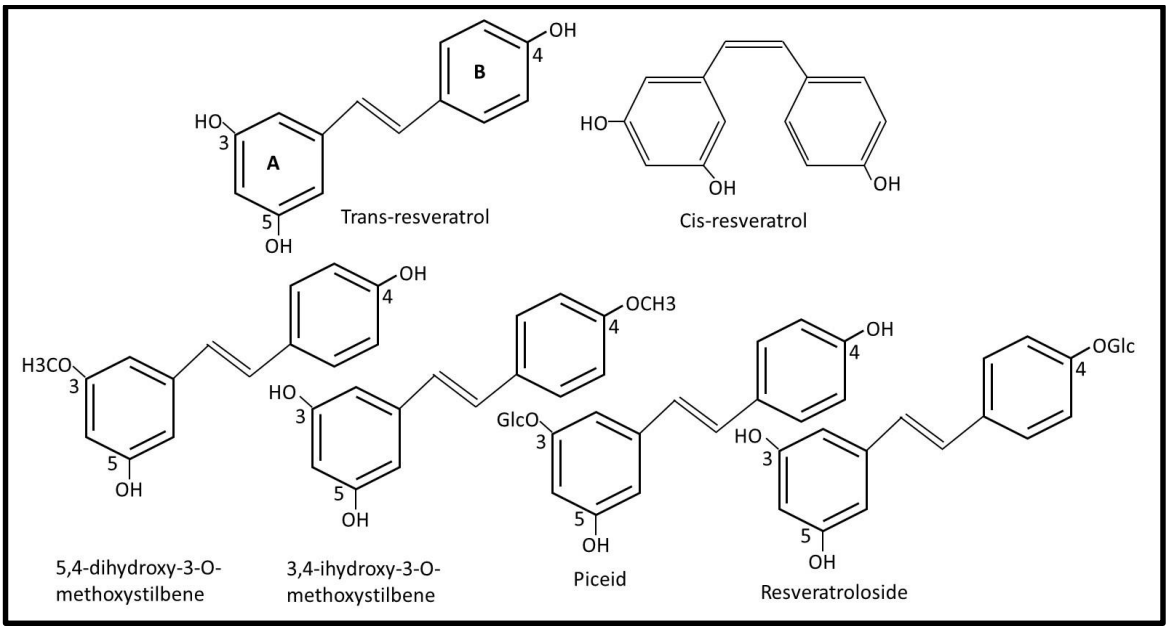


Figure 1. 3: The chemical structures of parental resveratrol comprising trans and cis resveratrol, methylated resveratrol and resveratrol glycone found in plant *tissue*.

1.1.3.2. Sources of resveratrol in the diet

Resveratrol is present in both edible and non-edible parts of some plants. The concentration of resveratrol in plant parts depends on various environmental stress factors including infection and ultra violet light exposure (Soleas et al., 1997; Stervbo et al., 2007). Resveratrol accumulates at high concentrations in red grape skin, and consequently it is relatively abundant in red wine (Wilfred, 2006). Resveratrol concentration in wine ranges between 0.1 and 15 mg for trans-resveratrol and 0.1 and 29 mg for tans-resveratrol-glucoside per litre (Frémont, 2000; Baptista et al., 2001; Moreno-Labanda et al., 2004; Mark et al., 2005). Other sources of resveratrol include peanut (Sobolev and Cole, 1999; Sanders et al., 2000; Chen et al., 2002; Akhtar et al., 2013) and the root of Japanese knotweed (Polygonum cuspidatum) which is a medical plant used particularly in Japan and China to treat conditions including cough and hypertension (Vastano et al., 2000). The concentration of trans-resveratrol in the edible parts of the peanut ranges between 0.02 and 7.8 µg per gram (Sales and Resurreccion, 2013) while the content per gram of *polygonum cuspidatum* root was measured to be 1653 µg of glycosylated trans-resveratrol and 523 µg of trans-resveratrol (Sales and Resurreccion, 2013). Resveratrol is present also in other plant products including grape berries, blueberries and bilberries (Lyons et al., 2003; Wang et al., 2013a) as well as pistachio nuts

(Sales and Resurreccion, 2013). It has been suggested that genetically modified organisms including bacteria, yeast and some plants can also provide a dietary source of resveratrol at sufficient concentrations to improve health (Jeandet et al., 2012).

1.1.2.3. Resveratrol absorption, metabolism and excretion

Routes for absorption, metabolism and secretion of resveratrol are very similar to those of genistein. Like genistein, the aglycosylated form of resveratrol is much more readily absorbed than glycosylated resveratrol (Meng et al., 2004) because relatively non polar (lipid soluble) aglycone can be absorbed passively by the intestinal epithelial cells (Yongmei et al., 2003; Velderrain-Rodriguez et al., 2014). Whereas glycosylated resveratrol undergoes deglycosylation by intestinal and bacterial β-glycosydase enzymes, it also appears to be absorbed through the sodium/glucose co-transporter-1 (SGLT1) (Sales and Resurreccion, 2013). Most resveratrol absorbed is conjugated with glucuronic acid and sulphate within the enterocytes and liver (Goldberg et al., 2003; Kaldas et al., 2003; Walle, 2011; Teng et al., 2012). As a result of these post-absorption modifications only a trace of untransformed resveratrol can be measured in the blood (Goldberg et al., 2003; Walle, 2011). Conjugated resveratrol may act as a pool to provide an active form of resveratrol to cells after deconjugation by tissue glucuronidase and sulfatase enzymes (Walle, 2011; Patel et al., 2013). Albumin and lipoproteins are important carrier molecules through which resveratrol may be delivered to cells thereby increase the half-life (Jannin et al., 2004). The half-life of resveratrol within the plasma was estimated to be about 7-14 h (Walle *et al.*, 2004). Primary routes of resveratrol excretion are through urine and bile (Walle et al., 2004). Like genistein, resveratrol excreted into the intestinal lumen in bile can be reabsorbed through the entero-hepatic circulation, which is thus an important route through which resveratrol is bioavailable (Marier *et al.*, 2002; Walle *et al.*, 2004) .

1.1.2.4. Resveratrol bioavailability

The rapid metabolism of resveratrol by liver and intestinal cells means that only traces of trans-resveratrol are found in the blood, hence it is essential to evaluate potential health benefits of resveratrol through in vivo studies (Asensi et al., 2002) as well as in vitro (where an advantage may be that specific molecular mechanisms are more tractable to perturbation and analysis). Two independent studies in human volunteers

revealed that absorption of parent molecule is relatively high while its bioavailability is very low as a result of its rapid metabolism (Goldberg et al., 2003; Walle et al., 2004). Furthermore, maximum concentrations reached appear to vary between different tissues. The highest concentrations have been measured in intestinal epithelial cells, stomach, liver, kidney and cardiac cells, whereas the lowest concentrations were reported in brain and testis (Vitrac et al., 2003; Wang et al., 2008a; Liang et al., 2013; Velderrain-Rodriguez et al., 2014).

Studies in animals showed that after intravenous injection of 20 mg of trans-resveratrol in the rabbit, the plasma concentration of resveratrol peaked (38.4-47.2 μM) within 5 minutes followed by a sharp reduction (0.7-1.1 μM) within an hour. The same pattern was observed in mouse and rat (Asensi et al., 2002). A study in human, rat and mouse showed that resveratrol (glycone and aglycone) was rapidly metabolised into glucuronated and sulphated forms, especially within the intestinal cells, after oral administration (Meng et al., 2004). Lipid nanoparticle technology has been developed with a view to increase resveratrol bioavailability and could potentially augment the likely health benefits of resveratrol consumption (Neves et al., 2013),

1.1.2.5. Health benefits of resveratrol

Like genistein, resveratrol has been the subject of substantial body of research aimed to determine if dietary consumption can deliver health benefits leading to claims that resveratrol may protect against conditions including cancer, oxidative stress and obesity. A critical overview of the research on this topic is provided below.

1.1.2.5.1. Anticancer effects of resveratrol

Like genistein, there has been extensive focus on the potential anti-cancer effects of resveratrol. Several studies have indicated that resveratrol may affect different stages of carcinogenesis including initiation, promotion and propagation (Sales and Resurreccion, 2013) by suppressing growth, proliferation, and inducing apoptotic pathways. These effects have been observed in a variety of different cancer cell types (Aggarwal et al., 2004; Khan et al., 2010; Lin et al., 2012a) including prostate (Harper et al., 2007; Seeni, 2009; Wang et al., 2010b; Sheth et al., 2012), breast cancer (Lee *et al.*, 2012b; Díaz-Chávez *et al.*, 2013), gastric cancer (Yang et al., 2013) and some others

(Aggarwal et al., 2004). Moreover, resveratrol (5 μM) inhibited the growth of B16 melanoma cells which is a highly invasive murine tumor cell model (Asensi et al., 2002).

There are several suggested mechanisms and pathways through which resveratrol may exercise anti-cancer activities. For example, resveratrol was demonstrated to interfere with tumor initiation via activation of the P53 pathway which is an important check point in detecting DNA damage before cells undergo further proliferation (Chow et al., 2010). Resveratrol was also shown to reduce the expression and activity of telomerase (hTLMA), specifically in cancer cells, by affecting the human telomerase reverse transcriptase (hTERT) subunit of the telomerase complex (Lanzilli et al., 2006). Moreover, resveratrol, like genistein, was found to reduce matrix metalloproteinase-9 (MMP9) expression and activity in BALB/C mouse and 4T1 cells, suggesting that it may also protect against tumor invasion and metastasis through stabilising cell to cell contacts (Lee *et al.*, 2012b). Other studies show that cancer protective effects of resveratrol may be mediated through up-regulation of microRNAs that target oncogenic genes (Tili et al., 2010; Qin et al., 2014). Resveratrol may also affect cancer cell properties through altering the DNA methylation status of cancer related genes, such as RASSF-1α (Zhu et al., 2012), which belongs to the RAS associated domain family and is potentially involved in tumor suppression (Clark et al., 2012).

1.1.2.5.2. Antioxidant effects of resveratrol

Like genistein, resveratrol is also a dietary agent with antioxidant activities that appear to be both direct and indirect in relation to ameliorating oxidative damage. The direct action of resveratrol as an anti-oxidant is by virtue of the free hydroxyl groups on its phenolic backbone and consistent with these predicted chemical properties resveratrol has been shown to act as a free radical scavenger (Murias et al., 2005; Hoshino et al., 2010). Resveratrol can also stimulate the activity (Lee et al., 2003) and the expression of anti-oxidant enzymes including glutathione peroxidase, catalase, hemeoxidase-1 and superoxide dismutase (Ungvari et al., 2007; Bujanda et al., 2008). Investigators demonstrated that resveratrol could protect primary coronary arterial endothelial cells from oxidative damage induced by UV-light and H_2O_2 (Ungvari et al., 2007). Additionally, resveratrol decreased oxidative damage in HUVEC cell exposed to H_2O_2. However, pro-oxidant actions including induction of free radical formation has also been measured and

found to be time, dose and cellular condition dependant. For example, malignant cells are more susceptible to be affected by the pro-oxidant effects of resveratrol than normal cells (Heiss et al., 2007; Sun et al., 2010).

1.1.2.5.3. Resveratrol and lifespan

A body of work has shown positive effects of resveratrol on lifespan particularly in model organisms including *S. cerevisiae* (Howitz et al., 2003), *C. elegans*, the fruit fly (*Drosophila melanogaster*) (Wood et al., 2004), fish (*Nothobranchius furzeri*) (Valenzano and Cellerino, 2006; Valenzano et al., 2006) and mice (Baur *et al.*, 2006). However, there are inconsistencies between studies. For example, mice fed a high-fat diet, which reduced lifespan, lived as long as mice which consumed a normal diet when the diet was supplemented with resveratrol while resveratrol did not affect the lifespan of those mice which fed a normal diet (Baur et al., 2006). Resveratrol has been reported to reduce features of aging such as aortic elasticity, inflammation and vascular endothelial damage (Pearson et al., 2008).

A possible explanation for the ability of resveratrol to extend lifespan in some systems is through the activation of the NAD-dependent histone deacetylase enzyme Sirt1 (Cherniack, 2010; Tollefsbol, 2010; Vetterli and Maechler, 2011). In respect to this activity, resveratrol may mimic some of the effects of dietary restriction, in which Sirt1 has a likely role (Guarente, 2013). However, this view has become the subject of vigorous challenge because of questions about whether resveratrol is a direct activator of Sirt1 or else its positive activations are a consequence of other beneficial effects of resveratrol (Kaeberlein *et al.*, 2005; Park *et al.*, 2012b). In addition, resveratrol has been reported to regulate the expression and activity of other age related genes including SIRT3, SIRT4 Forkhead box O (FOXO1, FOXO3a) and Pre-B cell colony enhancing factor (PBEF) (Das et al., 2011).

1.1.2.5.4. Other effects of resveratrol

In addition to anti-cancer, anti-oxidant activities and actions to extend life span several other beneficial effects of resveratrol that are consistent with the ability to promote health or extend life span have been documented. For example, resveratrol may stimulate mitochondrial biogenesis (Csiszar et al., 2009) and activity, mostly as a result of inducing PGC1α activity via activation of Sirt1 (Lagouge et al., 2006). Another effect of

resveratrol relates to anti-diabetic actions. It was reported that resveratrol can have insulin-like effects by reducing blood glucose (hypogycemic) and lipid (hypolipidemic) in streptozotocin-induced diabetic rats (Su et al., 2006; Thirunavukkarasu et al., 2007). Moreover, resveratrol appears to protect the vascular system through increasing the expression of the endothelial nitric oxide synthase (eNOS), which is responsible for protecting vascular integrity, in a dose and time dependent manner, as shown in human umbilical vein endothelial cells (HUVEC) (Wallerath et al., 2002). Resveratrol has also been reported to protect against neurodegenerative diseases including Alzheimer`s and Parkinson`s diseases (Sun et al., 2010). Other reported actions include prevention of platelet aggregation, thrombosis (Delmas et al., 2005) and inflammation (González et al., 2011).

1.1.2.6. Proposed mechanism of action of resveratrol

Of particular relevance to the current work, a significant body of knowledge is accumulating regarding diverse actions of resveratrol on lipogenesis. It is widely accepted that resveratrol can reduce the expression of peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer-binding protein-α (C/EBPα), which are the vital transcription factors for the initiation and maintenance of the process of lipogenesis by virtue of their roles in regulating the expression of lipogenic enzymes including fatty acid synthase, adipocyte protein-2 and lipoprotein lipase (Zhang et al., 2012). Resveratrol may also affect lipogenesis and lipid catabolism by mediating mitochondrial biogenesis and function via activation of PGC1α by Sirt1. The stimulatory effects of resveratrol on Sirt1 were demonstrated in both cell (3T3-L1) and animal (male FVB/N mouse) models by looking at the PGC1α acetylation status (Lin et al., 2002; Fischer-Posovszky et al., 2010; Lasa et al., 2012).

Other proposed mechanisms of action of resveratrol on lipogenesis relate to its inhibitory effects on the insulin signalling pathway. There is evidence that resveratrol can interact directly with and thus inhibit the insulin receptor, based on work in 3T3-L1 cells (Kwon et al., 2012). In the human hepatoma cell model (HepG2), resveratrol also has been reported to inhibit acetyl Co-A carboxylase activity, which is a key enzyme in the process of lipogenesis, through activation of the AMPK signalling pathway, (Zang et al., 2006). In addition, resveratrol was demonstrated to inhibit SREBP1c via the Sirt1/FOXO1 signalling

pathway, which was followed by a reduction in lipid biogenesis and accumulation in HepG2 cells and male Wistar rats (Bujanda et al., 2008; Wang et al., 2009).

1.2. Adipose tissue physiology

Obesity involves an increase in the mass of adipose tissue as a result of an increase in the level of fat accumulation within the cytoplasm of pre-existing adipocyte cells (adipocyte hypertrophy) and/or differentiation of new undifferentiated cells into adipocytes (adipocyte hyperplasia) (Ali et al., 2013). This process may include the synthesis and storage of ectopic fat within the cells of vital organs including the liver, which leads to impairment of their physiological performance (Morino et al., 2008). Obesity is one of the major health issues worldwide, and is associated with cardiovascular disease, type-2 diabetes (Poirier et al., 2006), decreased life expectancy (Blüher et al., 2003) and increased risk of cancer (Sorisky, 1999). The occurrence of obesity has increased over time due to multiple lifestyle and demographic factors, and it has been predicted that about 58% of the world`s population will be obese by 2030 (Kelly et al., 2008). Obesity is considered one of the main causes of death throughout the world and has been estimated to account for around 2.8 million deaths a year (Sorisky, 1999; WHO, 2012). Figure 4

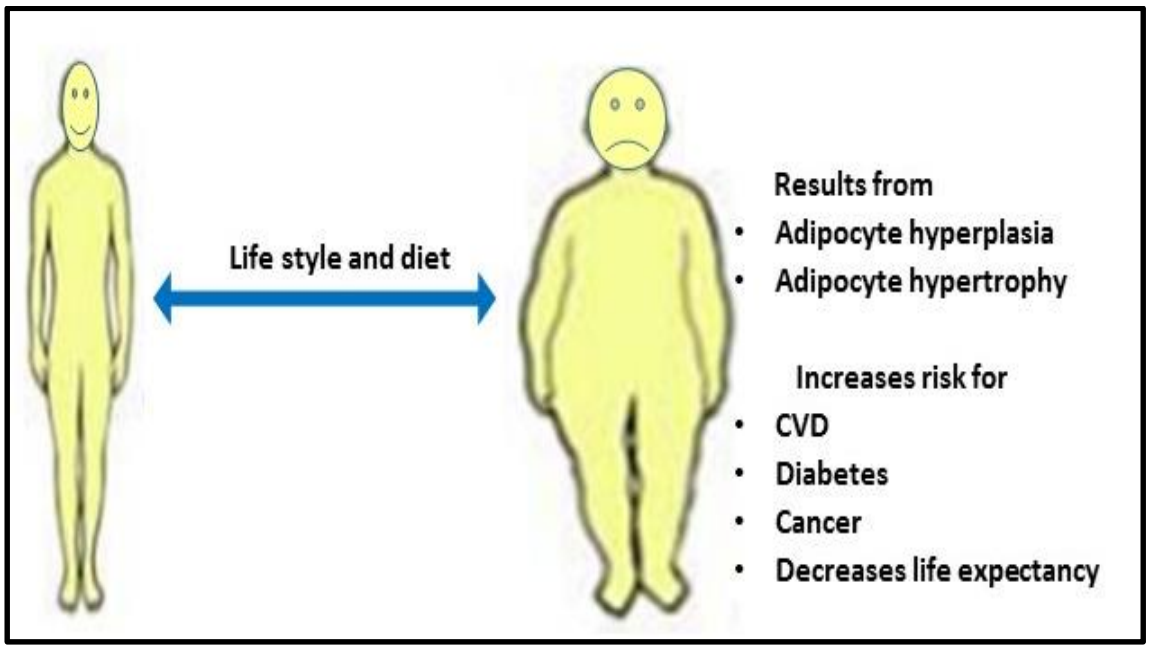


Figure 1. 4: Obesity is a dynamic process and it can be affected by life style and diet. It results from adipocyte hyperplasia and hypertrophy. It is also associated with an increase in the risk of cardiovascular disease (CVD), diabetes, cancer, and decreases life expectancy.

1.2.1. Adipocyte tissue phenotype and biochemistry

The two principal adipocyte phenotypes are white and brown adipocytes. These cells are distinct from each other morphologically and functionally. White adipocytes are involved in the process of lipogenesis and storage of fat while brown adipocytes have a role in thermoregulation and convert the proton electro chemical gradient of the mitochondrion into heat rather than ATP. The presence of brown adipocyte tissue is associated with lower risk of obesity and its related disorders.

1.2.1.1. White adipose tissue

White adipocytes synthesize and store fat in the form of triglycerides, and release it as a source of energy as required (Lafontan, 2005) depending on nutritional status. When energy availability is high, white adipocytes store energy in the form of triglycerides and release it in the form of free fatty acids during periods of energy deprivation (Sorisky, 1999). Initially white adipocyte tissue was considered to be primarily a passive repository for energy storage. However, with greater knowledge about the biochemistry of white adipose tissue has come the realisation that white adipocytes are highly active and responsive (Kim and Moustaid-Moussa, 2000), and actively communicate with other cells by receiving, synthesizing and sending biological signals in the form of biologically active peptides and proteins which are known as adipokines. Through adipokines, adipocytes play important roles in integrating and regulating the overall physiological and metabolic activity of adipose tissue and other organs (Frühbeck et al., 2001; Ali et al., 2013). The role of white adipose tissue in energy homeostasis is shown in Figure 5.

By serving as a specific repository for excess lipid white adipose tissue can be considered to have protective functions in terms of controlling lipotoxic damage stemming from high levels of circulating non-esterified fatty acids (NEFAs) (Unger, 2003) and decreasing ectopic fat accumulation (Boden *et al.*, 2005; Morino *et al.*, 2008). Consequently, these functions reduce inflammatory responses in metabolically active tissues including muscle, liver and brain (Szabo et al., 2005; Reyna et al., 2008; Wang et al., 2008c; Youssef-Elabd et al., 2012; Piya et al., 2013). Ectopic lipid biosynthesis and storage is associated with the development of several metabolic disorders, in particular diabetes mellitus type-2, non-alcoholic fatty liver disease and cardiovascular disease

(Anderson et al., 2003; Trujillo and Scherer, 2005; Kadowaki et al., 2006; Poirier et al., 2006; Morino et al., 2008).

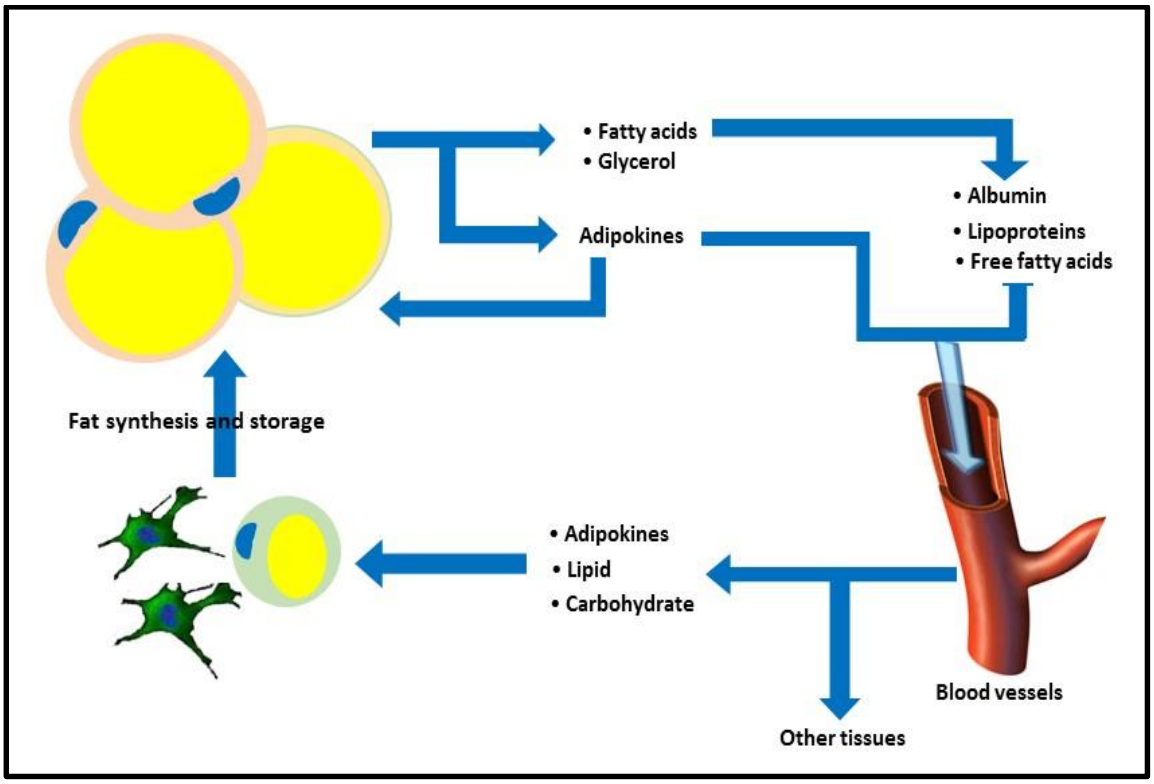


Figure 1. 5: the role of white adipose tissue in energy homeostasis. White adipocytes synthesize and store fat in the form of triglycerides and release it as a source of energy as required. The released lipid is transported in the blood as free fatty acids or by lipid carriers including albumin and/or lipoproteins. Adipokines, which are synthesised by white adipose tissue, have regulatory effects on the overall physiological and metabolic activity of adipose tissue and other organs.

1.2.1.1.1. White adipocyte differentiation

Adipogenesis is the process through which preadipocytes differentiate into mature adipocytes (Rosen and Spiegelman, 2000). It is a dynamic process (Spalding et al., 2008; Ali et al., 2013) affected by factors including the level of fat accumulation within cells (DiGirolamo et al., 1998; Cinti et al., 2005), stage of pregnancy, breast feeding and medication (Prins et al., 1997; Morroni et al., 2004).

Lipogenesis is a well-coordinated processes and is tightly regulated by co-operative interactions between several transcription factors including C/EBPs, PPARs (which belong to the ligand dependent nuclear hormone receptor superfamily) (Yeh et al., 1995; Mandrup and Lane, 1997; Brey et al., 2009; Ali et al., 2013) and SREBPs (a family of membrane bound transcription factors) (Shao and Espenshade, 2012). Actions of CCAAT/enhancer-binding proteins β/δ (C/EBPβ/δ) coordinate early stages of adipocyte differentiation (Cornelius et al., 1994), which is followed by up-regulation of C/EBPα and

PPARγ. Increased C/EBPα and PPARγ activity induces adipogenic genes leading to fat synthesis and accumulation within the cells (Rosen et al., 2002; Farmer, 2006).

In general, C/EBPα and PPARγ are considered the principle transactivators of lipogenesis-related genes (Sorisky, 1999; Tang and Lane, 1999; Rosen et al., 2002), and their expression is considered essential for the induction of white adipocyte differentiation and lipogenesis (Tang and Lane, 1999; Linhart et al., 2001; Koutnikova et al., 2003; Farmer, 2006; Lefterova and Lazar, 2009).

1.2.1.1.2. Genes that characterise white adipocytes

Numerous genes coding for enzymes and other proteins including adipokines with key roles in lipid metabolism are expressed selectively in white adipocytes and thus can be used as functional markers of this cell phenotype. Those of particular relevance to the current study are fatty acid synthase (FAS), acetyl Co-A carboxylase (ACC), adipocyte protein 2 (AP2), hormone sensitive lipase (HSL), chemerin and resistin.

1.2.1.1.2.1. ACC and FAS

Acetyl co-A carboxylase (ACC) and fatty acid synthase (FAS) are the key and rate-limiting enzymes in the process of fatty acid biosynthesis and lipogenesis. Acetyl Co-A carboxylase (ACC) catalyses the carboxylation of acetyl Co-A to malonyl Co-A, then fatty acid synthase (FAS) catalyses palmitate generation from malonal Co-A and acetyl Co-A as shown in Figure 6. The expression of both enzymes is highly upregulated at the early stages of lipogenesis and thus can be used as a cellular marker for commitment to the process of differentiation of precursor cells into white adipocytes (Hellerstein et al., 1996; Ameer et al., 2014).

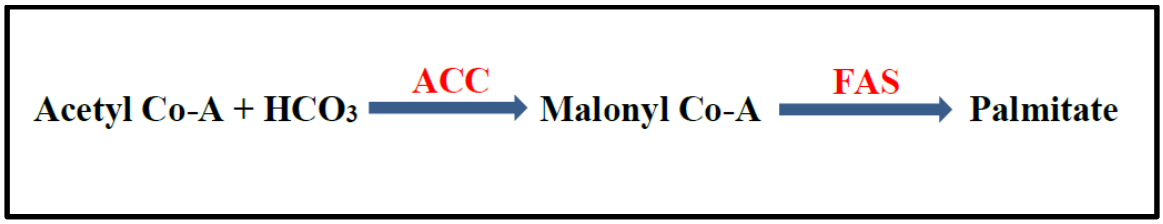


Figure 1. 6: The role of Acetyl Co-A carboxylase (ACC) and fatty acid synthase (FAS) in fatty acid synthesis. ACC catalyses the conversion of acetyl Co-A into malonyl Co-A, then FAS converts malonyl Co-A into palmitate.

Fatty acid synthase (approximately 260 kDa molecular weight) is a multifunctional cytosolic enzyme, encoded by a gene (*FASN*) located on the long arm of human

chromosome 17 (Jayakumar et al., 1994; Shah et al., 2006). There are two human ACC genes: *ACACA*, which is co-localised on the same chromosome as FAS, and *ACACB*, located on chromosome 12. They encode ACC-α (265 kDa) and ACC-β (280 kDa) respectively (Barber et al., 2005). In contrast to ACC-α, which is a cytosolic enzyme and involved in the process of lipogenesis, ACC-β is a mitochondrial enzyme and opposes mitochondrial β-oxidation by generating mitochondrial malonyl Co-A (Postic and Girard, 2008).

Regulation of these enzymes occurs at different levels. However, transcriptional regulation seems to be the predominant cellular mechanism through which cells control the long-term activities of these enzymes (Sul and Wang, 1998), and can be influenced by several factors including substrate availability, hormonal factors and developmental stage (Hillgartner et al., 1995; Sul and Wang, 1998). Transcription of both genes is under the control of lipogenesis-related transcription factors including SREBP1c, ChREBP, C/EBPα and PPARγ. Thus, any change in the expression and/or activities of these transcription factors affect the process of lipogenesis through modulating the expression of these key enzymes (Wu et al., 1999; Lefterova et al., 2008; Postic and Girard, 2008). Pharmacological inhibition of fatty acid synthase was found to be associated with a reduction in both food intake and marked loss of body weight in the mouse, whereas administration of an ACC inhibitor decreased fatty acid synthesis but did not affect these other variables (Loftus et al., 2000). Dysregulation of the expression and activity of these enzymes may be associated with pathological conditions including breast cancer; inhibition of FAS was found to be effective in reducing proliferation of cancer cells in rats and mice (Puig et al., 2009).

1.2.1.1.2.2. AP2 and HSL

Hormone sensitive lipase (HSL) is expressed at the early stages of white adipocyte differentiation during growth arrest of preadipocyte cells while adipocyte protein 2 (AP2) is expressed predominantly at the terminal stages of differentiation. AP2 and HSL have thus been recognised as markers of maturing preadipocytes (Spiegelman and Green, 1980; Amri et al., 1991; Sorisky, 1999). However, AP2 was shown to be expressed at a low level in adipocyte progenitor cells (Shan et al., 2013).

HSL is a cytosolic lipase enzyme and appears to be the key enzyme in the process of lipolysis (Yeaman, 2004; Arner and Langin, 2007). It is involved in fatty acylglycerol

hydrolysis (Thompson et al., 2010). In the human HSL gene (*LIPE*) is located on chromosome 19 (Kraemer and Shen, 2002) and it was found to be expressed at basal levels in several tissues including the supra-renal gland, macrophages, muscle cells, testis and pancreatic β cells (Khoo et al., 1993; Kraemer et al., 1993; Yeaman, 2004). HSL plays crucial roles in lipid biogenesis and enhances lipid accumulation within adipose tissue (Sekiya et al., 2004). Deletion of the gene in mice was associated with the development of a brown adipocyte phenotype (Osuga et al., 2000) and led to a significant increase in the expression level of UCP1 among white adipocyte depots (Ström et al., 2008).

Adipocyte protein 2 (AP2), also known as fatty acid binding protein 4 (FABP4), belongs to the family of intracellular lipid-binding proteins. The human gene (*FABP4*) is located on chromosome 8. AP2 is believed to act as a carrier protein for cytosolic fatty acids and it plays a role in lipid accumulation and distribution among cellular compartments including mitochondria, peroxisomes and the endoplasmic reticulum (Furuhashi and Hotamisligil, 2008; Thompson et al., 2009; Thompson et al., 2010; Scifres et al., 2011; Smathers and Petersen, 2011). High levels of circulating AP2 in obese mice was associated with obesity-related disorders including type-2 diabetes (Cao *et al.*, 2013b). However, mice lacking AP2 were protected from type-2 diabetes, non-alcoholic fatty liver and atherosclerosis (Hotamisligil, 2006; Furuhashi *et al.*, 2008). Inhibition of AP2 in mice using the pharmacological agent BMS309403 led to reduced symptoms associated with atherosclerosis and diabetes mellitus type-2 (Furuhashi et al., 2007). AP2 expression was down-regulated when Sirt1 was over-expressed in 3T3-L1 cells (Picard et al., 2004).

1.2.1.1.2.3. Chemerin and resistin

Chemerin and resistin are adipokines of particular relevance to the present study and have been used as markers of mature white adipocytes in other studies (Qiang et al., 2012; Sharp et al., 2012). Expression of both adipokines in brown adipocytes is very low (Steppan et al., 2001; Goralski et al., 2007) and appears to be down-regulated upon the induction of white to brown adipocyte inter-conversion (Seale et al., 2007; Qiang et al., 2012). Moreover, a direct correlation between chemerin and resistin expression was found in human adipose tissue (Lehrke et al., 2009).

The gene that encodes chemerin has been named both tazarotene-induced gene-2 (*TIG2*) and retinoic acid receptor responder-2 (*RARRES2*), and is located on human

chromosome 7 (Huang et al., 2010). Chemerin interacts with the chemokine-like receptor 1 (CMKLR1). It is involved in the inflammatory process where it acts as a chemoattractant to attract inflammatory cells into the site of injury (Ernst and Sinal, 2010). Chemerin is secreted predominantly by adipocytes and its presence seems to be essential in inducing adipocyte differentiation as its knockdown by siRNA in 3T3-L1 cells was sufficient to block differentiation programs (Goralski et al., 2007). A direct correlation between chemerin expression and body weight was observed in obese human subjects compared with lean subjects (Roman *et al.*, 2012). The serum level of chemerin was found to be abnormally high in obese-diabetic mice and humans (Stejskal et al., 2008; Ernst et al., 2010). Chemerin appears to increase the risk of obesity related disorders including insulin resistance in both obese human subjects and primary human skeletal muscles (Sell et al., 2009; Roman et al., 2012).

Resistin, or adipocyte secreted factor (ADSF), is related to a group of proteins known as resistin-like molecules. The gene (*Retn*) is located on human chromosome 19 (Jamaluddin et al., 2012). Expression of resistin correlates with the process of lipogenesis. Its knockdown by siRNA in differentiated 3T3-L1 cells ameliorated fatty acid β-oxidation and reduced lipogenesis as a result of down-regulation of carbohydrate responsive element binding protein (ChREBP) and its downstream genes (fatty acid synthase and acetyl Co-A carboxylase) (Ikeda et al., 2013). High levels of circulating resistin have been found to have detrimental effects on the development of cardiovascular diseases, diabetes mellitus type-2 and other obesity related disorders (Filková et al., 2009). Furthermore, a direct link between resistin and insulin resistance has been established in both humans and rodents (Schwartz and Lazar, 2011).

1.2.1.2. Brown adipose tissue

In contrast to white adipose tissue, the presence of brown adipose tissue reduces fat storage. Brown adipose tissue functions metabolically to dissipate energy in the form of heat (Lowell and Spiegelman, 2000). Brown adipocytes are characterised by having small multi-locular fat droplets (Cinti *et al.*, 1997; Ravussin and Galgani, 2011; Lee *et al.*, 2012c), and the cytoplasm of brown adipocytes is densely packed with mitochondria. The mitochondria of brown adipocytes can be distinguished from those of white adipocytes by the presence of uncoupling protein-1 (UCP1) which is responsible for dispersing stored

energy as heat by uncoupling ATP synthesis from electron transport (Matthias et al., 2000; Cannon and Nedergaard, 2004; Frontini et al., 2007). Absence of or defects in UCP1 activity in brown adipose tissue have been seen to be associated with obesity, metabolic disorders (Nedergaard *et al.*, 2007; Cannon and Nedergaard, 2009) and defective adaptive thermogenesis (Golozoubova et al., 2001; Meyer et al., 2010) in humans and mice.

The ectopic presence of brown adipocytes in the skeletal muscle of transgenic mice protected them against metabolic disorders (Almind et al., 2007). In addition, the induction of the expression of genes that are characteristically expressed in brown adipocytes in the murine white adipocyte cell line models, 3T3-L1, was demonstrated to be associated with a sharp reduction in the expression level of white adipocyte marker genes including *Retn* (resistin) (Seale et al., 2007), *Rarres2* (chemerin) (Qiang et al., 2012) and *Acaca* (acetyl Co-A carboxylase) (Kamiya et al., 2012). It appears that the quantity of brown adipose tissue is sex dependent (Rodríguez-Cuenca et al., 2002). Women appeared to have more functional brown adipose tissue than men as measured using positron emission tomography-computed tomography (PET/CT) (Cohade et al., 2003; Yeung et al., 2003; Cypess et al., 2009).

A strategy to prevent obesity and its related disorders may thus be to increase brown adipose tissue mass, which could be achieved by stimulate browning of white adipocytes or encouraging the differentiation of adipocyte precursor cells along lineage with a brown adipocyte phenotype.

1.2.1.2.1 Classification of brown adipocytes

Brown adipocytes can be divided into classical brown and beige/brite (brown-in-white fat) adipocytes. Classical brown and beige adipocytes are distinguished by distinct gene expression profiles and their embryological origin (Giralt and Villarroya, 2013). As already noted, the basal expression level of UCP1 is particularly high in classical brown adipocytes. However, beige adipocytes express UCP1 at high levels only under specific conditions, in particular with β-adrenergic stimulation or exposure to a cold (Waldén et al., 2011; Harms and Seale, 2013). The unique set of genes expressed in beige adipocytes includes CD-137, which can be used to distinguish beige adipocytes from other cells (Sharp et al., 2012; Wu et al., 2012).

Classical brown adipocytes originate from the same precursor cells as myocytes, characterised by the expression of Myf5 which is a myogenic transcription factor (Timmons et al., 2007; Seale et al., 2008; Kajimura et al., 2009). In contrast, beige adipocytes have the same origin as white adipocytes (Himms-Hagen et al., 2000; Granneman et al., 2005; Petrovic et al., 2010; Wu et al., 2012). However, it is not clear whether beige adipocytes originate from trans-differentiation of white adipocytes or from other undifferentiated cells within white adipose tissue repositories (Rosenwald et al., 2013; Wang et al., 2013c), as illustrated in Figure 7.

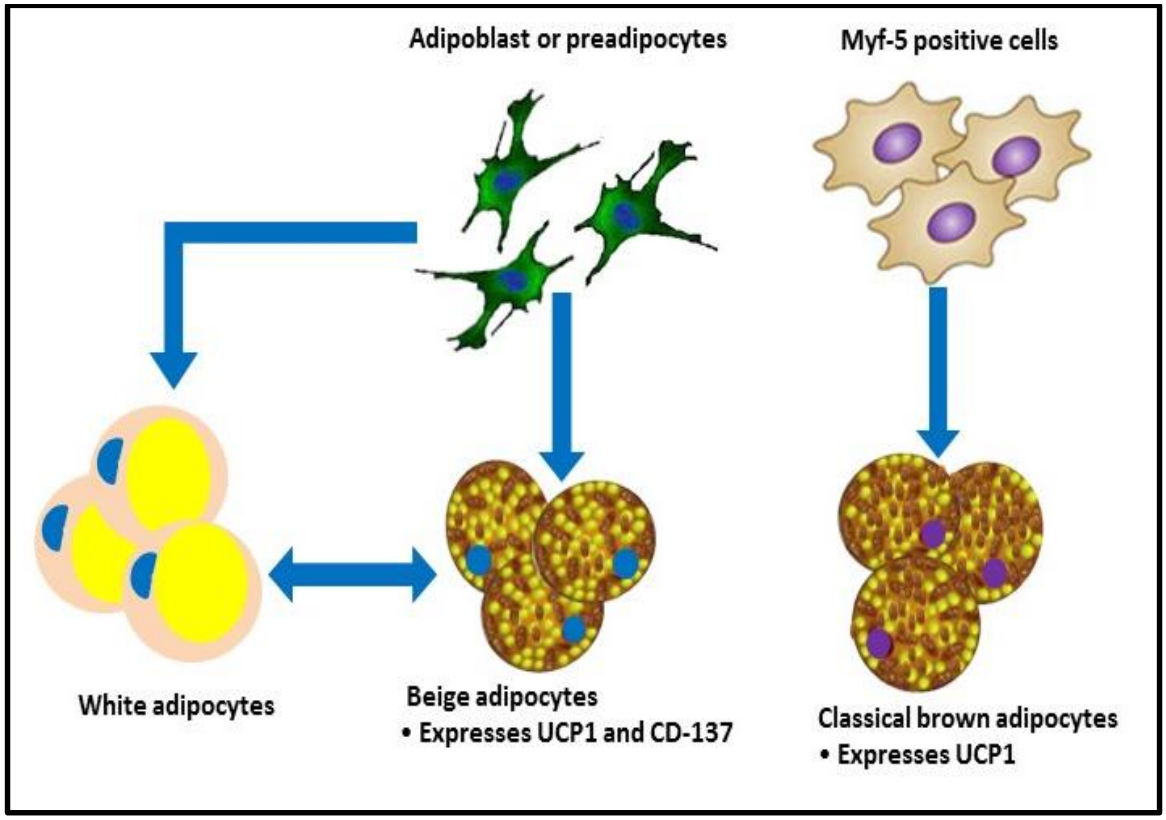


Figure 1. 7: Origins of brown adipocytes. Classical brown adipocytes originate from Myf-5 positive cells, Beige adipocytes transdifferentiate from mature or undifferentiated white adipocyte precursor cells. The presence of brown adipocytes in body is associated with low body weight, decreases risk of cardiovascular disease, diabetes and metabolic disorders, besides increasing adaptive thermogenesis.

1.2.1.2.3. Thermogenesis

The control of body temperature is achieved largely as a result of cellular metabolic activity that generates heat, and adapts to regulate body temperature in response to environmental and physiological changes (Mrozek et al., 2012). ATP is the main source of energy, which is generated as a result of electron transfer from reduced

form of nicotinamide adenine dinucleotide (NADH) or Flavin adenine dinucleotide (FADH) to oxygen to generate water through a chain of protein complexes comprising NADH oxidoreductase (complex I), succinate-Q reductase (complex II), cytochrome bc1 (complex III) and chytochrome c oxidase (complex IV). Ubiquinone (coenzyme Q) and cytochrome c shuttle electrons between complex I, complex II, complex III and complex IV. A proton-gradient is generated within mitochondrial intermembrane space and mitochondrial matrix as a result of protons being pumped through complex I, complex III and complex IV from the mitochondrial matrix into the intermembrane space as electrons are transported. In the absence of UCP1, protons return into the mitochondrial matrix through ATP synthase complex, thus driving ATP synthesis (Berg *et al.*, 2002). However, in response to a sudden and/or chronic change in the environmental temperature or in response to physiological factors such as hormonal stimuli or disease alternative thermogenic pathways, including shivering and non-shivering thermogenesis, are invoked.

Shivering thermogenesis is an action of the skeletal muscle (Block, 1994; Golozoubova et al., 2001) to maintain body temperature within the physiological range by increasing cellular metabolic activity (Meyer et al., 2010; Cannon and Nedergaard, 2011). However, this response is transient and cannot be sustained for a long period of time due to muscular damage and/or exhaustion (Bal et al., 2012). Non-shivering thermogenesis generates heat as a result of proton transport across the inner mitochondrial membrane by uncoupling protein-1, thereby dissipating the proton from electro-chemical gradient and uncouples mitochondrial electron transport ATP synthesis, which leads to energy being released as heat (Golozoubova et al., 2001; Golozoubova et al., 2006). This process is shown in Figure 8.

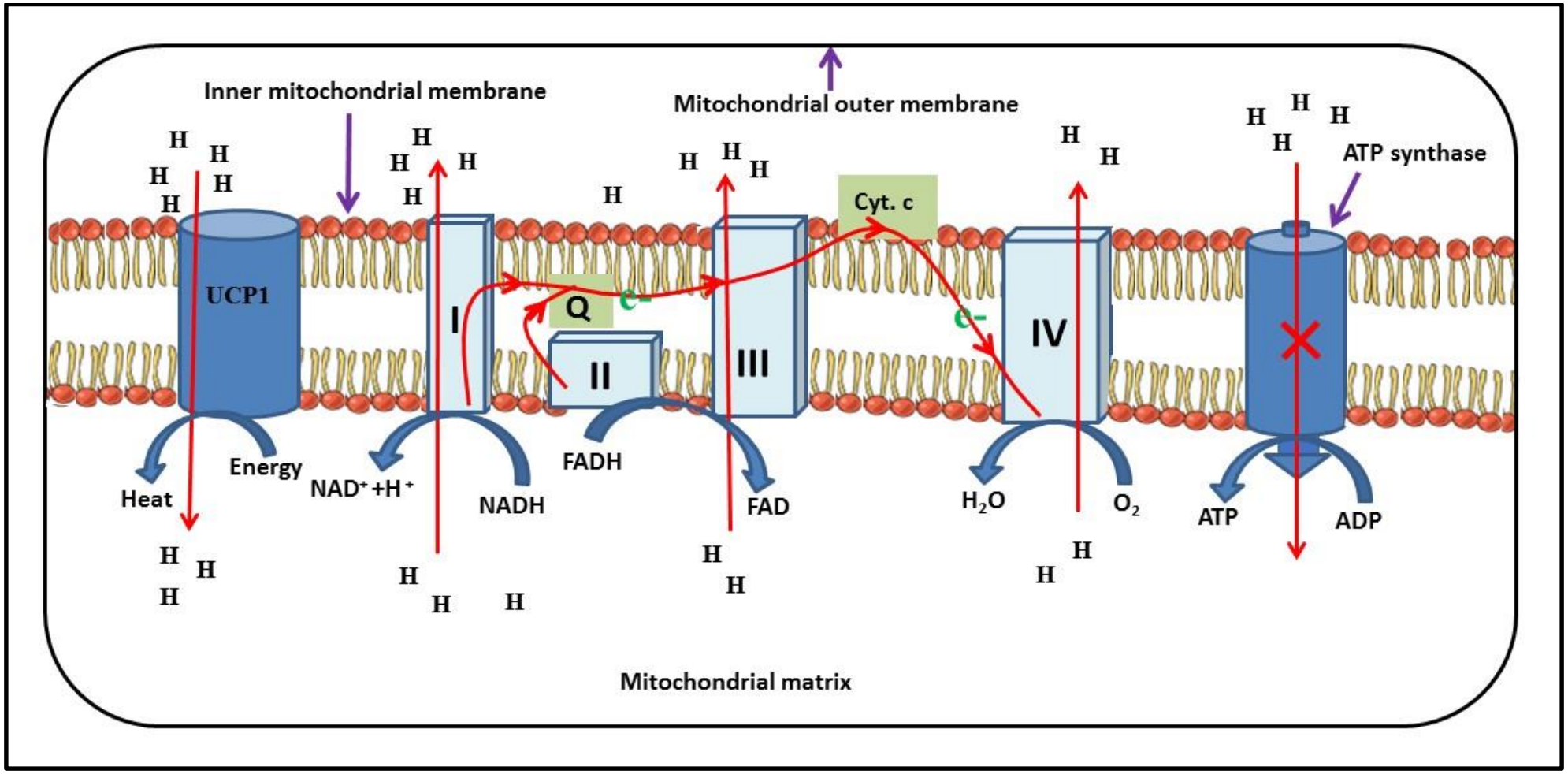


Figure 1. 8: Non-shivering thermogenesis. Proton transport across the inner mitochondrial membrane by UCP1 leads to dissipation of the mitochondrial proton electro-chemical gradient. The gradient is generated as a result of proton transport from the mitochondrial matrix into the mitochondrial intermembrane space as electron transport occurs through complex I, complex III and complex IV. Electron transport is thus uncoupled from ATP synthesis and the energy released as heat.

1.2.1.2.4 Anatomical distribution of brown adipose tissue

Human infants almost certainly have relatively large depots of brown adipose tissue compared with adults (Gilsanz et al., 2013). The process of shivering thermogenesis does not contribute substantially to heat generation in human neonate. Thus, brown adipose tissue is particularly important for maintaining the body temperature (Ravussin and Galgani, 2011). It was a longstanding view that the human adult has no brown adipose tissue (Barrington and Maisey, 1996; Engel et al., 1996). However, positron emission tomography (PET) after administration of flourodeoxyglucose and histological studies have more lately found that adult humans can have substantiated depots of brown adipose tissue regardless of age (Hany et al., 2002; Yeung et al., 2003; Zingaretti et al., 2009) mainly distributed in distinct regions including the neck and supraclavicular region, with small depots being detected in the mediastinum, paravertebral and perinephric regions of the body (Nedergaard et al., 2007; Sacks and Symonds, 2013). It remains unclear if these depots found in adult humans are truly brown adipocytes or if they would be more accurately described as beige adipocytes (Sharp et al., 2012).

Brown adipose tissue in rodents is located principally within the axillary, interscapular, periaortic and perirenal regions of the body (Lee et al., 2014).

1.2.1.2.5 Genes that characterises brown adipose tissue

Brown adipocytes are characterised by expressing a distinct set of genes that are involved in the process of differentiation or confer the specialised function of non-shivering thermogenesis. Additionally, these genes are a useful research tool since they can be used to distinguish brown adipocytes from other cell types and to distinguish between classical brown and beige adipocytes. The genes that can be used to identify brown adipocytes include uncoupling protein-1 (UCP1) and CD-137.

1.2.1.2.5.1. UCP1

Uncoupling protein 1 belongs to the UCP superfamily that also in turn includes UCP2 and UCP3, which are specific mitochondrial membrane anion transporters. UCP1 is the product of a single gene (*UCP1*), which is located on human and mouse chromosomes 4 and 8 respectively. The UCP1 gene consists of 8 exons and codes for protein with a molecular weight 32-23 kDa across species (Mozo et al., 2005). The promoter and

different distal enhancer regions of the UCP1 genes include several putative sites for binding of transcription factors that play important roles in adipose cell physiology. These include two C/EBP binding sites, a PPRE (peroxisome proliferator activated receptor response element), a TRE (thyroid hormone response element), and a RARE (retinoic acid response element). Activation at all of these sites appears to be important in the transcriptional activation of UCP1 (Yubero et al., 1994; Rim and Kozak, 2002; Cannon and Nedergaard, 2004; Karamanlidis et al., 2007).

Knockout of UCP1 in mice (Kontani *et al.*, 2005; Feldmann *et al.*, 2009) was associated with obesity and cold sensitivity. In contrast, only minor deficiencies in non-shivering thermogenesis were observed in UCP2 and UCP4 knockout mice (Mozo et al., 2005). Thus, UCP1 appears to be the protein primarily responsible for the induction of non-shivering thermogenesis (Golozoubova et al., 2001; Golozoubova et al., 2006). In agreement, over expression of UCP1 in an adipocyte-specific transgenic mouse model (Kopecky et al., 1996) was associated with a lean phenotype and protection against high-fat-diet-induced obesity with improved blood lipid profile (Rossmeisl et al., 2005). Similarly, ectopic expression of UCP1 in the liver of a dietary-induced mouse model of obesity was associated with reduced body weight and reduction in obesity-related disorders including hyperphagia and insulin resistance. In addition, the activity of the key lipogenic enzyme acetyl Co-A carboxylase was reduced (Ishigaki et al., 2005). Consistent with these in vivo studies overexpression of UCP1 in 3T3-L1 cells reduced lipid content and affected lipid distribution such that fat droplets had the paucilocular appearance that is the characteristic of brown adipocytes (Si et al., 2007).

UCP1 activity has been found to be affected by factors including nutritional status specific diet components and genetic background. Palmitate and retinoic acid were found to activate and induce UCP1-dependent thermogenesis in both primary mouse brown adipocytes and mitochondria from rat brown adipose tissue (Shabalina et al., 2008; Divakaruni et al., 2012). Ephedrine and caffeine also stimulated expression of UCP1 in both brown and white adipose tissue depots of obese human subjects (De Matteis *et al.*, 2002). A single nucleotide polymorphism (SNP) at the transcriptional start site of the human UCP1 gene was found to reduce UCP1 expression (Esterbauer et al., 1998; Rose et al., 2011). Furthermore, carriers of this SNP had a reduced response to dietary induced

thermogenesis compared with non-carriers (Nagai et al., 2003) and were more susceptible to obesity (Sramkova et al., 2007).

1.2.1.2.5.2. CD-137

CD-137 is a member of the tumor necrotic factor receptor family (alternatively known as TNFRSF9). It plays important roles in the regulation of cellular mediated immunity (Melero et al., 2008) and has recently been discovered to be selectively expressed in beige rather than classical brown adipocytes (Wu et al., 2012; Fu et al., 2014). The expression of CD-137 led to the classification of some brown adipocyte depots including human epicardial and visceral paracardial fat depots as beige rather than classical brown adipose tissue (Sacks et al., 2013).

1.2.1.2.6. Mediators of brown adipocyte differentiation

Several enzymes and transcription factors including Sirt1, C/EBPβ and PGC1α, appear to be involved in the process of white to brown interconversion in response to environmental and physiological stimuli. These factors have been defined as mediators of white to brown adipocyte transdifferentiation. However, the contribution of these factors in this process is complex and likely to be affected by several other cellular and molecular factors.

1.2.1.2.6.1. Sirt1

Sirt1 is an enzyme that modifies proteins by NAD-dependent lysine deacetylation and it is well-preserved among species. Mammalian cells express seven functionally distinct sirtuins, Sirt1 to Sirt7. Sirt1, which is a mammalian homolog of yeast Sirt2, is involved in several vital processes including inflammatory and metabolic pathways (Revollo and Li, 2013; Davenport et al., 2014). Sirt1, yeast Sirt2 and homologes in other species have been proposed as mediators of the lifespan extension observed in response to dietary restriction. Sirt1 expression was decreased with age in the liver of old mice (Jin et al., 2011) and life expectancy was improved upon its activation (Mitchell et al., 2014). In addition, pharmacological activation of Sirt1 was reported to have several health-beneficial actions including improvement in the blood lipid profile and amelioration non-alcoholic fatty liver (Mitchell et al., 2014) and diabetes (Qiao and Shao, 2006).

Sirt1 expression and activity have been reported to change in response to several factors including low energy intake (Walker et al., 2010), exercise (Suwa et al., 2008), and some dietary agents such as genistein (Rasbach and Schnellmann, 2008), resveratrol (Lagouge et al., 2006; Rodgers et al., 2008; Fernandez-Marcos and Auwerx, 2011; Price et al., 2012) and α-lipoic acid (Fernández-Galilea et al., 2015).

Previous studies have shown that Sirt1 expression was reduced in obese humans and mice (Guarente, 2013), while its over-expression in mouse adipocyte depots and in the 3T3-L1 adipocyte model led to a dramatic reduction in the expression level of the key regulatory factors of lipogenesis CEBPα and PPARγ in parallel with reduced fat accumulation. In agreement, expression of CEBPα and PPARγ was increased when Sirt1 expression was reduced using siRNA (Picard et al., 2004). Also Sirt1 knockdown in mice exacerbated the progression of non-alcoholic fatty liver which was coincident with an increase in the expression level of lipogenic enzymes comprising acetyl Co-A carboxylase and fatty acid synthase (Wang et al., 2010a). It also reduced activity of PPARα, a key activator of fatty acid β-oxidation. These responses were mediated through inactivation of PGC-1α (Purushotham et al., 2009).

Sirt1 appears to act as a co-repressor of PPARγ through docking with PPARγ cofactors including nuclear receptor co-repressor (NCoR), and silencing mediators of retinoid and thyroid hormone receptors (SMRT) (Picard et al., 2004). Moreover, miRNA-146b, which targets Sirt1 mRNA, was showed to enhance adipogenesis via increasing PPARγ expression as a result of an increase in the acetylation level of FOXO1 (Ahn et al., 2013). In vitro, Sirt1-mediated deacetylation of PPARγ was reported to be followed by activation of brown adipocyte genes. Thus PPARγ has dual effects on lipogenesis and whether it activates expression of genes that contribute to the white or brown phenotype depends on its acetylation status (Qiang et al., 2012).

Sirt1 appears to have pivotal roles in the development of beige adipocytes as an increase in the expression of Sirt1 in a transgenic mouse model which increased the expression level of brown adipocyte marker genes, including *Ucp1* and *Cebpb*, among white adipose tissue depots while the white adipocyte marker genes, *Rarres2* (chemerin) and *Retn* (resistin), were found to be reduced. However, the expression of *Ucp1* and

Cebpb in classical brown adipocytes was shown to be unaffected by increased expression or Sirt1 knock-down in transgenic mice (Qiang et al., 2012).

The activities of Sirt1 and PGC1α are closely linked (Aquilano et al., 2010). An increase in the expression level of Sirt1 affected by knockdown of miR-32a, an endogenous miRNA against Sirt1 mRNA, enhanced the browning of white adipocytes which was coincident with PGC1α deacetylation in mouse and 3T3-L1 cells (Fu et al., 2014). PGC1α is a key stimulating factor for mitochondrial biogenesis which occurs at a high level during the development of brown adipocytes (Rodgers et al., 2005; Guarente, 2007; Fernandez-Marcos and Auwerx, 2011).

1.2.1.2.6.2. C/EBPβ

C/EBPβ is a member of C/EBP (CCAAT/ enhancer binding protein) family of basic leucine zipper transcription factors, which consists of six distinct proteins that play prominent roles in several vital cellular processes including the cell cycle (Tsukada et al., 2011), cell differentiation (Calkhoven et al., 2000) and metabolic homeostasis (Roesler, 2001). C/EBPβ is a positive regulator of Sirt1 (Jin et al., 2011).

C/EBPβ has recently been recognised as a conspicuous player in the development of classical brown and beige adipocytes, and has been used as a characteristic marker of brown adipocytes (Qiang et al., 2012). Its expression appears to be critical for the differentiation of brown adipose tissue in mice (Tanaka et al., 1997). In addition, an overexpression of C/EBPβ in human skin fibroblasts, mouse skin fibroblasts and 3T3-L1 cells was shown to be sufficient to convert these cells into brown adipocytes (Karamanlidis et al., 2007; Kajimura et al., 2009). Likewise, knock-down of C/EBPβ in parallel with PRDM16 (positive regulatory domain contain 16) overexpression in C2C12 myoblast cells was arguably sufficient to prevent differentiation of these cells into brown adipocytes by as measured using UCP1 and PGC1α expression as markers. Consistently, the C/EBPβ deficient mouse embryo was reported to have depleted brown adipose tissue and caused a reduction in the expression level of UCP1 (Kajimura et al., 2009). In addition, defective thermogenesis was observed in the C/EBPβ knockout mouse (Carmona et al., 2005). Replacement of C/EBPα with C/EBPβ in mouse led to the development of a lean phenotype (Chen et al., 2000) with higher energy expenditure as a result of an increase in the expression level of UCP1 within white adipocyte depots (Chiu et al., 2004).

Observations concerning miRNA-155, which is an endogenous microRNA against C/EBPβ mRNA, also provide supporting evidence that C/EBPβ is important for the development of beige adipose tissue. Specifically, the presence of miRNA-155 was negatively correlated with both classical brown and beige adipocyte phenotypical changes. Moreover, deletion of miRNA-155 in the mouse was effective in inducing the browning of white adipocytes as indicated by the gene expression profile (Chen et al., 2013b).

It was suggested that the action of C/EBPβ may be through binding to the cAMP response element (CRE) within the promoter of genes including the PGC1α genes that confer the brown/beige adipocyte phenotype. However, later work has shown that activation of the PGC1α promoter by CEBPβ was mediated through a sequence which was separate from the CRE and has been termed the C/EBPβ response element (Wang et al., 2008b).

1.2.1.2.6.3. PGC1α

Peroxisome proliferator activated receptor gamma coactivator 1 α (PGC1α) belongs to a family of transcriptional co-activators that in turn includes PGC1β and PGC1-related co-activators. PGC1α is involved in the regulation of several metabolic pathways including glycolysis, gluconeogenesis, remodelling of brown and white adipocytes as well as fat metabolism (Puigserver and Spiegelman, 2003). PGC1α is expressed mainly in tissues that are very active metabolically including skeletal muscle and brown adipose tissue (Finck and Kelly, 2006). PGC1α is a central factor in regulating mitochondrial biogenesis and activity (Lin et al., 2002; Austin and St-Pierre, 2012) by controlling the functions of nuclear respiratory factor 1 and 2 (NRF1 and 2), which control the transcription of mitonuclear respiratory complex proteins and the mitochondrial transcription factor A (TFAM) (Santos and Kowluru, 2011). The N-terminus of PGC1α associates with histone deacetylation complexes including steroid receptor coactivator-1 (SRC1) and CREB binding protein/P300 (Finck and Kelly, 2006). Thus there is a view that PGC1α has histone remodelling activity and makes DNA available for transcription factors to transcribe target genes.

PGC1α undergoes several posttranslational modifications that modulate its function, specifically acetylation (Lagouge et al., 2006; Gerhart-Hines, 2007),

phosphorylation, ubiquitination and methylation (Fernandez-Marcos and Auwerx, 2011). The functional effects of PGC1α acetylation to inhibit activity are well-defined. Sirt1 is one of the enzymes that can deacetylate PGC1α. So this is likely to be one of the main actions of Sirt1 through which its effects to protect against features of aging are mediated (Rodgers et al., 2005; Lagouge et al., 2006; Gerhart-Hines, 2007). Acetylation of PGC1α by general control non-repressed protein-5 (GCN5) during conditions of high energy availability represses its activity (Gerhart-Hines, 2007; Fernandez-Marcos and Auwerx, 2011). It also prevents its association with the transcriptional complexes it forms with C/EBPβ and PPARγ to stimulate the differentiation of brown adipose tissue (Fu et al., 2014).

Activation of PGC1α by Sirt1 enhances mitochondrial biogenesis (Pereira et al., 2012). Likewise, the activation results in a sharp increase in the expression level of enzymes that catalyse β-oxidation, specifically medium chain acyl-co-A dehydrogenase, carnitine acyltransferase and carnitine palmitoyl transferase both in vivo and in vitro including C2C12 cell line, mouse embryonic fibroblast (MEFs), primary mouse muscle cells, HepG2 cells and in mice (Kim et al., 2004; Kim et al., 2005; Gerhart-Hines, 2007).

Consistent with the PGC1α being a key orchestrator of brown adipose tissue development (Uldry *et al.*, 2006; Harms and Seale, 2013), overexpression in human primary subcutaneous white adipocytes and the mouse white adipocyte model (3T3F442A) promoted expression of UCP1 and mitochondrial biogenesis (Puigserver et al., 1998; Tiraby et al., 2003). Also consistent with PGC1α having an important role in brown adipose tissue development, the PGC1α null mouse was seen to have a defective thermogenesis and poorer survival under cold condition (Lin et al., 2004). The stimulatory effect of PGC1α on the UCP1 expression appears to be mediated through recruitment of PPARγ and the thyroid receptor onto the UCP1 promoter (Puigserver et al., 1998; Tiraby et al., 2003). In the same way, PGC1α acts as a coactivator of PPARα to increase UCP1 expression (Barberá et al., 2001) and it also associates with the retinoic acid receptor to augment UCP1 expression (Liang and Ward, 2006).

1.2.1.2.7. Molecular mechanisms that regulate genes expressed in brown adipocytes

The classical mechanism of activating the expression of genes that are key determinants of the activity of brown adipose tissue is through the activation of β-adrenergic receptors (Cannon and Nedergaard, 2004; Mattsson et al., 2011). This is, then, followed by the activation of adenylyl cyclase which triggers an increase in the level of cAMP that in turn activates protein kinase A. this process lead to the activation of cAMP response element binding protein (CREBP), which activates genes that are under the control of cAMP response element (CRE) including UCP genes and PGC1α (Cannon and Nedergaard, 2004).

Tri-iodothyronine (T3) binds to and activates thyroid receptor β (TRβ), which is a nuclear receptor. Subsequently, it stimulates brown adipocyte differentiation through inducing genes under the control of thyroid response element (TRE). T3 also increases the sensitivity of the β-adrenergic stimulated pathways of CRE mediated gene activation. In addition, brown adipose tissue type 2 deiodinase (D2) expression is up-regulated by T3 providing a feed-forward activation mechanism to generate T3 from thyroxine (T4), the inactive form of thyroid hormone (Martinez de Mena et al., 2010). Figure 9 shows the interactions between the β-adrenergic and T3-mediated pathways through which genes acting in brown adipocyte development are stimulated.

The mechanisms switching on the gene expression profile that characterises beige adipocytes are not well-understood but several hypothetical models have been suggested in this regards. Plausible and well-rehearsed proposed mechanisms include the involvement of Sirt1 either by deacetylating and thus activating PPARγ (Qiang et al., 2012) or PGC1α (Fu et al., 2014). Downstream of the latter activity, PGC1α, can act as a coactivator of PPARα and may act in concert with retinoic acid receptor to induce brown adipocyte phenotypical changes (Barberá et al., 2001; Liang and Ward, 2006). C/EBPβ may also stimulate the expression of UCP1 through binding to the cAMP response element (CRE) and/or C/EBPβ response element (C/ERE) within the promoter (Wang et al., 2008b; Chen et al., 2013b). Binding of PRDM16 (positive regulatory domain contain 16) with PPARγ and/or C/EBPβ, with subsequent activation of the genes that characterises brown

adipocytes also appears to be important in brown adipocyte differentiation (Seale et al., 2008; Kajimura et al., 2009).

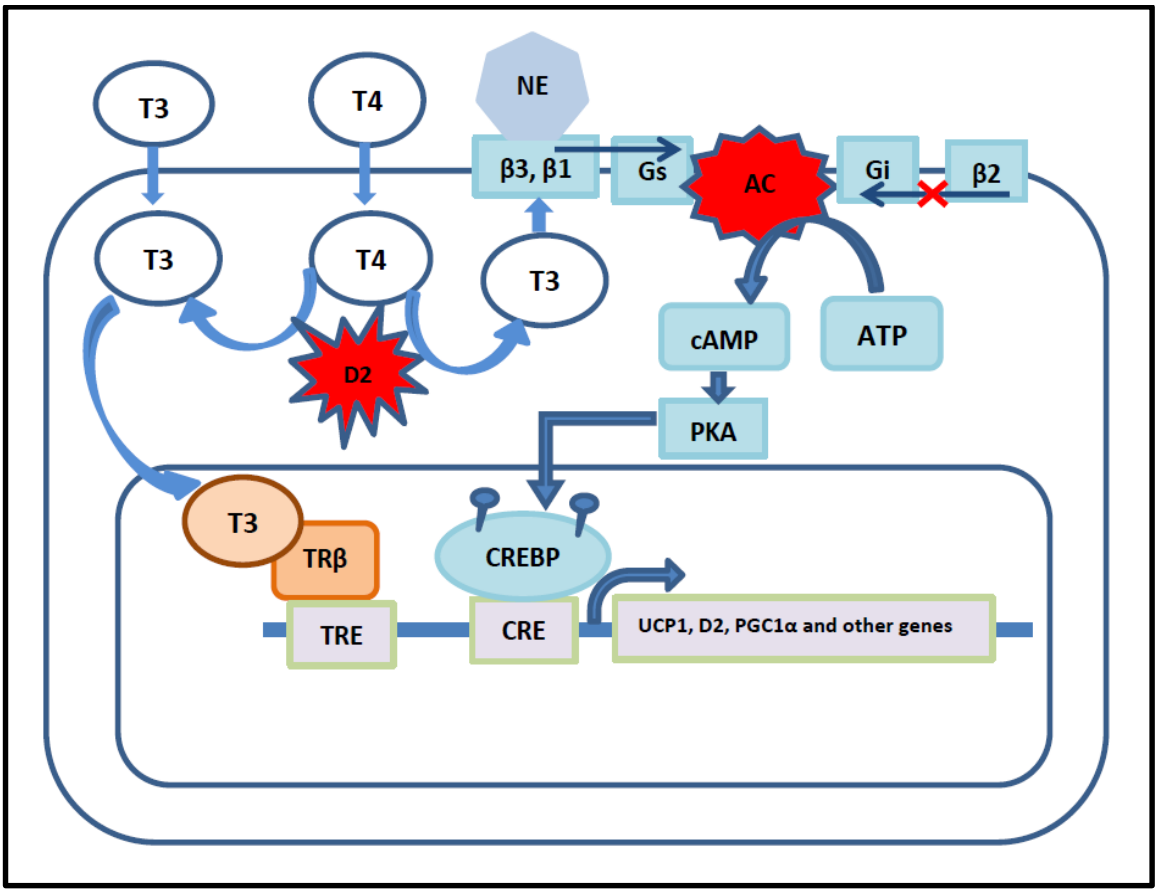


Figure 1. 9: Effects of β-adrenergic receptor (β3 and β1) activation and T3 on brown adipocyte gene expression. Following activation of β-adrenergic receptors (β1 and β3) with adrenergic receptor activators like nicotine amide (NE), adenylyl cyclase (AC) is activated. The active adenylyl cyclase catalyses conversion of ATP into cyclic-adenosine monophosphate which is followed by protein kinase-A (PKA) activation leading to activation of the cyclic adenosine monophosphate response element binding protein (CREBP) by phosphorylation. The activated CREBP enhances the expression of genes under the control of cyclic adenosine monophosphate response element (CRE) including UCP1 and PGC1α. Thyroid receptor activation by tri-iodothyronine (T3) triggers UCP1, PGC1α and D2 expression through the thyroid response element (TRE). T3 can also be generated from thyroxine (T4) by type2 deiodinase (D2). The B2-adrenergic receptor is a negative regulator of AC.

1.2.1.2.8. External factors that affect brown adipocyte development

External influences on the development of beige adipose tissue are not well understood (Collins et al., 2010). The development and maintenance of beige adipose tissue appear to be a dynamic process (Frontini and Cinti, 2010) and may be affected by environmental, physiological and dietary factors including exposure to cold, adrenergic stimulation (Cinti, 2009; Barbatelli et al., 2010), energy intake (Cinti, 2011), exercise

(Bostrom et al., 2012), hormonal status (Quarta et al., 2012) and micronutrient availability (Fernández-Galilea et al., 2015).

A body of work has shown that exposure to cold in humans and rodents was associated with an increase in the mass of brown adipose tissue, which generally has been assumed to have resulted from trans-differentiation of pre-existing white adipocytes into brown adipocytes (Huttunen et al., 1981; Murano et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Bostrom et al., 2012; Vitali et al., 2012). Also, adrenergic stimulation in the rat was found to increase the amount of brown adipocyte-like cells among white adipocyte depots characterised by accumulation of paucilocular fat droplets and an increase in the mitochondrial content of the cells (Himms-Hagen et al., 2000). High temperature has been reported to have the opposite effects of cold temperature and enhance brown to white adipose tissue interconversion in rats (Cancello et al., 1998).

A high fat diet also was reported to promote the expression of genes that characterise brown adipocytes including UCP genes, in both brown and white adipocyte depots in mouse (Rippe et al., 2000). Such a diet led to improved thermogenesis in human subjects (Nagai et al., 2003). In contrast, fasting reduced the expression of UCP1 in the brown adipose tissue of rat (Champigny and Ricquier, 1990). In general, however, there is a paucity of knowledge about the effect of diet on brown adipose tissue development; thus research in this area is required (Fromme and Klingenspor, 2010).

Effects of hormonal status on brown adipose tissues are revealed by several studies. For example, ovariectomized mice gained weight in parallel with a reduction in the expression level of UCP1 within brown adipose tissue (Pedersen et al., 2001). In another study on mice, progesterone and testosterone were found to have the opposing effects on UCP1 expression in brown adipose tissue. Expression was stimulated by progesterone but inhibited by testosterone (Rodríguez et al., 2002). Also, estrogen substitution in ovariectomized rats was found to stimulate UCP gene expression in both brown and white adipose tissue depots (Pedersen *et al.*, 2001).

Some lines of evidence suggest that a brown adipocyte phenotype could be achieved by manipulating the transcriptional pattern of target cells through ectopic expression of specific transcription factors or genes responsible for phenotypic features of brown adipocytes. For example, over expression of Sirt1 (Qiang et al., 2012), PGC1α

(Tiraby et al., 2003), C/EBPβ (Karamanlidis et al., 2007; Kajimura et al., 2009), PRDM16 (Seale et al., 2007) and UCP1 (Ishigaki et al., 2005) have all been observed to induce features of the brown/beige adipocyte phenotype. Similarly, deletion or knock down of some genes that contribute to white adipocyte physiology including HSL (Osuga et al., 2000; Ström et al., 2008) and fat specific protein 27 (FSP27), which participates in fat droplet formation (Tanaka et al., 2014), was sufficient to reprogram white adipocytes into brown-like adipocytes in transgenic mice.

It is likely that epigenetic mechanisms, such as DNA methylation, histone modification and miRNAs, play roles in determining adipocyte fate. These processes could affect events such as recruitment of transcription factors to regulate gene expression profiles that confer the brown adipocyte phenotype. At present, however, detailed knowledge about how epigenetic modification affects adipocyte physiology is lacking (Sugii and Evans, 2011). The influential role of miRNAs in determining the phenotype of adipocytes is however being uncovered. For instance, the presence of miRNA-155, which is an endogenous anti C/EBPβ, was sufficient to interfere with the development of primary mouse brown adipocytes and the differentiation of brown adipocytes from white adipocytes, while deletion or reducing its expression counteracted these effects (Chen et al., 2013b). Others found that knock-down of miRNA-32a, which is an endogenous miRNA against Sirt1, promoted white to brown adipocyte interconversion as a result of an increase in the expected deacetylation and activation of PGC1α in mice and 3T3-L1 cells (Fu et al., 2014).

Alpha-lipoic acid (100-250 μM), which is an antioxidant dietary compound (Durrani et al., 2010), was also revealed to induce browning of white adipocytes through its stimulatory effects on the expression and activity of Sirt1, which was followed by an increase in mitochondriogenesis and an augmented expression level of UCP1, in a human cell model of fully differentiated subcutaneous preadipocytes (Fernández-Galilea et al., 2015).

Rosiglitazone, a PPARγ agonist, was also found to enhance mitochondrial biogenesis and UCP1 expression in mouse white adipose tissue (Sell et al., 2004; Bogacka et al., 2005; Petrovic et al., 2008).

1.2.1.2.9. Effects of genistein and other polyphenols on UCP1 expression and mitochondrial activity

Genistein was reported to restore mitochondrial function and biogenesis in both cell and animal models including cerebral ischemia in a mouse model, rat primary neuronal cell culture (Adams et al., 2012; Qian et al., 2012), , T47D (a breast cancer cell model) (Pons et al., 2014) and rabbit primary renal proximal tubular cells (Rasbach and Schnellmann, 2008). A likely mediating mechanism is up-regulation of Sirt1 and PGC1-α expression (Rasbach and Schnellmann, 2008). Likewise, genistein may indirectly regulate Sirt1 activity through inducing AMP-activated PK (AMPK) which increases the intracellular level of the Sirt1 activator NAD. These effects of genistein on AMPK have been observed under normal and inflammatory conditions in both cell and animal models including rodents, 3T3-L1 and prostate cancer cells (Hwang et al., 2005; Cederroth et al., 2008; Park et al., 2010; Wang et al., 2013b). In addition, PGC1α phosphorylation by AMPK appears to be important for subsequent PGC1α deacetylation by Sirt1 (Canto et al., 2009; Fernandez-Marcos and Auwerx, 2011). It has been shown that genistein can prevent mitochondria mediated cellular apoptosis either via down-regulation of Bax, which is a pro-apoptotic protein factor interfering with mitochondrial function by disturbing mitochondrial outer membrane permeability (Adams et al., 2012), or as a result of genistein`s antioxidant activities (Qian et al., 2012).

Genistein (5-50 mg/Kg per a day) was also found to increase UCP1 expression in white adipose tissue depots in mice (Penza et al., 2006). Moreover, the obesogenic effects of high caloric diet in mice were diminished in the presence of genistein (0.2%, 0.4% genistein for 12 weeks) through stimulating an increase in the expression of PGC1α, mitochondrial medium chain acyl Co-A dehydrogenase, UCP2, and mitochondrial biogenesis (Lee et al., 2006).

Several other studies have confirmed the beneficial effects of dietary isoflavones on mitochondrial functionality, particularly upregulation of UCP protein. For example, shorter-term (14 days) and chronic (180 days) exposure of rats to soya protein (which contains isoflavone including genistein and diedzein) reduced body weight and energy expenditure besides increasing UCP expression in brown and white adipose tissue (Takahashi and Ide, 2008; Torre-Villalvazo *et al.*, 2008). Similarly, soya protein (5000 mg

isoflavones/Kg of diet) alone and/or in combination with exercise in rats stimulated the expression of PGC1α and PPARα, and reduced the lipid content of the liver as well as epididymal adipose tissue (Morifuji et al., 2006).

Treatment of rats for 14 days with black soya bean skin extract (0.2, 1 and 2% of diet), which is a rich source of polyphenols including epicatechin and procyanidin, was reported to prevent dietary induced obesity by stimulating fat catabolism through increasing the UCP1 and UCP2 expression in both brown and white adipose tissue depots (Kanamoto et al., 2011). Furthermore, high UCP1 expression and oxygen consumption rate was reported in rodents treated with isoflavone-rich plant extract (Puerariae thomsonii) and soya based diets (Lephart *et al.*, 2004; Kamiya *et al.*, 2012).

Diadzein (50 mg per kilogram body weight), which is present in soyabeans together with genistein, was found to invoke UCP1 expression and prevented an increase in the body weight (Crespillo et al., 2011). Similarly, resveratrol (30 mg/Kg/day for 6 weeks) was also demonstrated to increase UCP expression and mitochondriogenesis in brown adipocytes and muscle cell of rats (Alberdi et al., 2013).

1.2.1.3. Effects of genistein on adipocyte physiology

Various reported effects of genistein both in vivo and in vitro are commensurate with an effect on fat metabolisms. However, the molecular mechanism through which genistein affects fat metabolism is not understood fully. The impact of genistein on lipid metabolism appears to be dose and time dependant. In vitro, lower doses of genistein (up to 1 μM) were reported to inhibit lipogenesis, and the effect appeared to be estrogen receptor-dependent since it was reversed in the presence of the estrogen receptor antagonist ICI 164,382. However, higher concentrations of genistein (>10 μM) were observed to stimulate lipogenesis in primary mouse bone marrow and KS483 cells through a mechanism that appeared to be estrogen receptor-independent (Dang et al., 2003). Similarly, 50 μM genistein stimulated lipogenesis in primary human synovial fibroblasts (Relic et al., 2009). Conversely, in vivo in the mouse a lower dose of genistein (50 mg/Kg per a day) enhanced lipogenesis while high dose of genistein (200 mg/Kg per a day) reduced lipid accumulation (Penza et al., 2006).

Overall, however, it appears that genistein has the potential to affect lipid biogenesis in a beneficial manner either by inhibiting the process of lipogenesis and/or

through improving the process of lipolysis and fat metabolism. In support of this view the expression of key transcription factors and their downstream genes involved in the process of lipid biogenesis, including PPARγ, liver X receptor (LXR) and retinoid X receptor-α, declined when genistein was included (2000 or 4000 mg genistein/Kg) in a diet that induced obesity in mice (Kim et al., 2010). Also, genistein was observed to interfere with the expression and activity of sterol regulatory element binding protein-1 (SREBP-1) (Kim *et al.*, 2010), which is a membrane bound transcription factor involved in the regulation of the lipid biogenesis (Horton et al., 2002) through its action to down-regulate site-1 protease (S1P) (Shin et al., 2007). SIP is a serine protease and is required for SREBP-1 to be activated and translocated into the nucleus (Espenshade, 2006). Genistein (50 μM) also reduced fat synthesis and accumulation in 3T3-L1 cells by decreasing the expression level of C/EBPα and its downstream genes including fatty acid synthase (Zhang et al., 2009), and possibly by decreasing glucose uptake via its direct inhibitory action on the transmembrane glucose transporter GLUT4 (Bazuine et al., 2005).

Treatment of obese Zucker rats with genistein led to a reduction in the blood triglyceride and cholesterol level and decreased fat accumulation within the liver (Mezei *et al.*, 2003). Similarly, male Sprague-Dawley rats exposed to genistein (0.03, 0.3, or 1.2 mg/day) for 5 days during the neonatal period were protected against high fat diet induced non-alcoholic fatty liver and body weight gain, consistent with an observed increase in the β-oxidation indicated by an increase in the expression level of PPARα (Huang *et al.*, 2011). Also, genistein (10-100 μM) prevented fat accumulation within the cytoplasm of HepG2 cells and there was an increase in the level of β-oxidation indicated by an increase in the expression level of carnitine palmitoyltransferase 1 (CPT1) and PPARα (Kim *et al.*, 2004). The effect of genistein on fat catabolism, particularly in the context of fatty liver, may be mediated through PPARα (Kim *et al.*, 2004; Kim *et al.*, 2005) and its downstream genes including carnitine palmitoyltransferase and very long chain acyl Co-A dehydrogenase (Kim et al., 2005; Kim et al., 2010).

Genistein was also found to enhance PGC1α expression (Lee et al., 2006), consistent with mitochondrial biogenesis (Puigserver *et al.*, 1999; Liang and Ward, 2006), UCP1 expression (Neve et al., 2000; Liang and Ward, 2006) and fatty acid β-oxidation (Lee

et al., 2015). Another regulatory effect of genistein on lipid metabolism may be related to its up-regulatory effect on Sirt1 expression (Rasbach and Schnellmann, 2008).

1.2.1.4. Effects of resveratrol on adipocyte physiology

Extensive studies have shown that resveratrol can modulate the lipogenic process and reduce lipid accumulation in adipocyte tissue. Similar to reported actions of genistein, resveratrol has been demonstrated to interfere with or reduce several lipid-related health problems including non-alcoholic fatty liver, and to improve the plasma lipid profile by reducing the expression levels and/or activities of transcription factors and enzymes with roles in lipid biogenesis. For example, resveratrol was shown to reduce adipocyte proliferation and differentiation in preadipocytes taken from patients with Simpson Golabi-Behmel syndrome (SGBS), which is an over-growth syndrome (Fischer-Posovszky et al., 2010). Another study demonstrated that resveratrol was able to prevent hepatosteatosis in HBV (hepatitis B virus) X protein transgenic mice and HepH2 cells co-incident with a reduction in the activity and expression of the liver X receptor-α (LXRα). LXRα increases expression of sterol regulatory element-binding protein-1c (SREBP-1c), leading to a reduction in the expression of lipogenic enzymes including FAS and ACC (Lin et al., 2012b; Jin et al., 2013). Similarly, the supplementation of a high fat diet with resveratrol for 60 days reduced body fat mass, improved the blood lipid profile and reduced plasma insulin concentration in male mice and reduced the expression level of PARP-γ and SREBP-1, while stimulating Sirt1 expression (Andrade *et al.*, 2014b). In contrast, dietary resveratrol (30 mg/kg body weight/day for 6 weeks) in rats was found to have no effect either on the mRNA of lipogenesis-related transcription factors (SREBP1c and PPARγ) or their downstream genes (FAS, ACC, LPL); however, the activities of FAS and ACC were significantly reduced (Alberdi *et al.*, 2011).

1.3. Estrogen receptors

Estrogen receptors belong to the steroid/thyroid superfamily of intracellular receptors (Evans, 1988). There are two principle estrogen receptors (ERs), ERα (Green et al., 1986) and ERβ (Kuiper et al., 1996), which are products of distinct genes. The ERα gene is located on human chromosome-6 and the ERβ gene is on human chromosome-14 (Krzystyniak, 2002; Herynk and Fuqua, 2004). Each ER gene consists of eight exons separated by seven intronic regions (Ascenzi et al., 2006). Due to the use of alternative promoters ERs are expressed as different variants depending on tissue and cellular conditions (Li et al., 2000; Hirata et al., 2001; Ascenzi et al., 2006). ERs show wide distribution throughout the body tissues and are involved in physiological and pathophysiological process including growth, development, adipogenesis and cancer (Dunnwald *et al.*, 2007; Lee *et al.*, 2012a).

1.3.1. Structure of estrogen receptors

Estrogen receptors have three main domains: the N-terminal domain (A/B-domain), DNA binding domain (C-domain) and the ligand binding domain (D/E/F-domain) (Beato, 1989). The detail of ER is shown in Figure 10

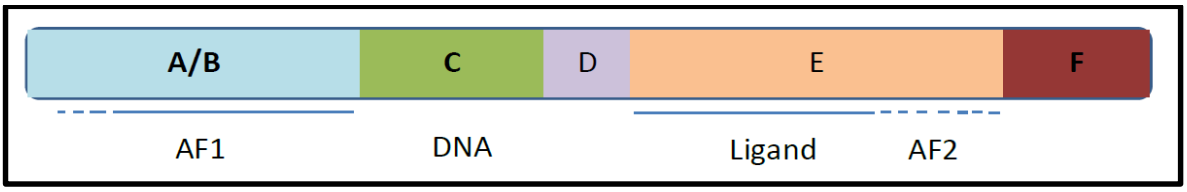


Figure 1. 10: Domains of the estrogen receptor that shows the N-terminus domain (A/B), DNA binding domain (C), hinge domain (D), ligand binding domain (E) and F-domain. Regions responsible for activation (AF-1 and AF-2) are also indicated.

ERα and ERβ show extensive similarity and are highly conserved particularly in the DNA-binding domain (Mosselman et al., 1996; Kuiper et al., 1997) and ligand-binding domains. The main variability between the receptor subtypes is confined to the hinge domain (D-region), F-region and N-terminal domain (Mosselman et al., 1996).

Each of the ERs domains has a specific function. The D/E/F domain is involved in processes including nuclear localization, binding with ligands and heat-shock proteins, dimerization and transactivation (Tsai and O'Malley, 1994), while the C-domain, the DNA

binding domain, contains two zinc fingers, which play a crucial role in recognising and binding to the estrogen response element (ERE) of target genes (Ascenzi et al., 2006).

ERs have two domains known as activation function domains (AFs). AF-1 is a part of A/B region at the N-terminus of the ER while AF-2 is towards the C-terminus. The AF-1 domain can activate gene expression in the absence of ligand when it is phosphorylated through the action of kinase enzymes as a result of extracellular stimuli (Marino et al., 2006). However, AF-2 domain responses are mainly ligand dependent and full activation of both receptor types requires ligand-dependent interaction between AF-1 and AF-2 domains. It was demonstrated that an agonist ER ligand stimulates fully activation of the ER while antagonist agents have the opposite effect by preventing the interaction between these domains as a result of inducing conformational changes in receptor structure (Kumar and Thompson, 2003).

1.3.2. Estrogen receptor isoforms

Several isoforms of both ERα and ERβ result from use of alternative promoters or alternative splicing (Moore et al., 1998; Ascenzi et al., 2006). Approximately, 20 isoforms of ERα (Poola et al., 2000) and 5 isoforms of ERβ (Moore et al., 1998) have been detected. Among ERα isoforms ERα66, ERα46 and ERα39 are well-characterised (Reid *et al.*, 2002; Chen *et al.*, 2014). ERα46 is distinguished from the wild type by lacking the AF-1 domain (Li et al., 2003) while ERα36 is devoid of both AF (AF-1 and AF-2) regions (Wang et al., 2005). ERβ isoforms (ERβ 2, 3, 4, and 5) are characterised by having a shorter C-terminus compared with ERβ1. Functionally, they are inactive on their own but they become active upon dimerization with ERβ1 (Leung et al., 2006). Furthermore, ERβ2 can form a heterodimer with ERα to confer a negative regulatory function (Omoto et al., 2003).

The expression of these isoforms appears to be cell type and cell condition dependent. For instance, ERβ3 was expressed only in testis, and ERβ1 was absent or its expression was reduced in liver and colon tumor (Moore *et al.*, 1998; Campbell-Thompson *et al.*, 2001; Thomas and Gustafsson, 2011).

1.3.3. Distribution of estrogen receptors

ERα and ERβ are present together in several tissues including brain, the reproductive system, the skeletal system, heart and adipose tissues. However, their expression ratio varies in different tissue types and under different conditions (Paech et

al., 1997; Pearce and Jordan, 2004; Böttner et al., 2014). For example, it was demonstrated that ERβ is the predominant ER in colon tissue while in hepatic tissue ERα is the prominent receptor (Pearce and Jordan, 2004; Böttner et al., 2014). The expression profiles of ERs can be disturbed under pathophysiological conditions. For instance, the expression of ERβ was dramatically reduced in colon adenocarcinoma (Konstantinopoulos et al., 2003) and prostate cancer (Leav et al., 2001).

1.3.4. Mechanism of estrogen receptor responses

In the classical pathway, ER ligands enter the cell through simple or facilitated diffusion (Tsai and O'Malley, 1994). Upon binding to the receptor, they induce a structural change (Beekman et al., 1993) causing the receptor to translocate from the cytoplasm into the nucleus of the cell (Beato, 1989) where it binds directly to the estrogen response element (ERE) (Tsai and O'Malley, 1994) as a dimer (Schwabe et al., 1990). The core ERE consensus sequence occurs frequently as a series of repeats separated by three nucleotide intervals (Glass, 1994; Schwabe et al., 1995; Vanacker et al., 1999). In the absence of the activating ligand, ERs can bind with heat-shock proteins (heat-shock protein-90 and heat-shock protein-70) and in this form, they appear to be inactive (Tsai and O'Malley, 1994; Ascenzi et al., 2006). It has recently been shown that in the absence of estrogen, ERα (unliganded) can bind to several sites within the genome where it appears to have a role in controlling and stabilizing the expression of these genes (Caizzi et al., 2014).

In the non-classical pathway the action of the activated ER does not involve binding to the ERE but it is through the ER binding with other transcription factors including activator protein-1 (AP-1) and specificity protein-1 (SP-1). The activated ER and AP1/or SP1 complex then binds to sequences including AP-1 and GC-rich SP1 sites to regulate gene expression (Kim et al., 2003; DeNardo et al., 2005; Burek et al., 2014). There is evidence that the AF-1 and AF-2 ER motifs are crucial for this non-classical pathway response (Kim et al., 2003; Achari et al., 2009).

ERs can function as heterodimers or homodimers (Paulmurugan *et al.*, 2011). As heterodimers, ERα and ERβ can activate different sets of estrogen responsive genes (Monroe *et al.*, 2005) and can have opposite effects to homodimers. For example, an ERα homodimer stimulated proliferation of normal murine mammary epithelial cells (HC11)

(Helguero *et al.*, 2005) whereas the ERα/ERβ heterodimer was seen to have the opposite effect (Powell *et al.*, 2012). Homodimers of ERs appear to have agonist and/or antagonist action on estrogen responsive genes depending on the type of the ligand and the promoter elements. For instance, binding of 17β-estradiol with ERα activated transcription of a reporter plasmid construct that included an AP1 (activated protein 1) element, whereas ERβ had the opposite effect. In contrast, ERβ with a tamoxifen ligand enhanced the transcriptional activity of the same construct (Paech et al., 1997).

ERs can also be activated via ligand-independent pathways that involve their phosphorylation in the nucleus by kinase enzymes including mitogen activated protein kinase (MAPK) and protein kinase A (PKA) (Marino *et al.*, 2006; Morani *et al.*, 2008; Cao *et al.*, 2013b). There is evidence that activation of this pathway can be through the epidermal growth factor receptor (Nilsson et al., 2001) or membrane bound estrogen receptors (Razandi et al., 2004).

17β-estradiol and other agents with estrogenic action may have classical nuclear ER-independent actions by activating G-protein-coupled receptor-30 (GPR30), which is a non-classical membrane G-protein-coupled ER, within the plasma and endoplasmic reticulum membranes (Filardo *et al.*, 2002; Revankar *et al.*, 2005; Chevalier *et al.*, 2012; Wei *et al.*, 2014) and mostly associated with non-genomic estrogen signalling (Olde and Leeb-Lundberg, 2009). .

1.3.5. Estrogen receptors and cancer

ERs status can provide important prognostic information about some types of cancer (Herynk and Fuqua, 2004; Dunnwald *et al.*, 2007). Based on epidemiological studies, ERβ was identified as an important indicator to classify severity and stage of colon cancer (Rudolph et al., 2012). A substantial reduction and/or absence of ERβ is characteristic of some forms of cancer including colon adinocarcinoma (Foley et al., 2000; Campbell-Thompson et al., 2001; Konstantinopoulos et al., 2003), ovarian cancer (Rutherford et al., 2000; Bossard et al., 2012), breast cancer (Skliris et al., 2003; Huang et al., 2014), prostate cancer (Leav et al., 2001) and lung cancer (Kawai et al., 2005).

Overexpression of ERβ can inhibit proliferation of both ERα positive and negative human ovarian cancer cell lines (BG1 and PEO14) (Bossard et al., 2012). Similarly, overexpression of ERβ reduced cell proliferation and induced cell cycle arrest at G1 in

colon cancer cell models (SW480 and HCT-116) (Hartman et al., 2009). Human breast cancers expressing high levels of ERβ have been shown to respond better to hormonal therapy than ERβ negative breast cancers (Murphy et al., 2002). Furthermore, ERβ was found to counteract the proliferative effects of ER-α in the breast cancer cell model T47D and in rat prostate cancer (Ström et al., 2004; Attia and Ederveen, 2012). However, the ERβ knockout mouse showed abnormal growth of breast and prostate epithelial tissue cells, which is a predisposing factor in neoplasia formation (Gustafsson and Warner, 2000; Weihua et al., 2001). Again, ERβ knockdown by siRNA in a prostate cancer cell model (LNCaP) increased the proliferative rate of the cells in association with a sharp increase in the expression level of ER-α (Stettner et al., 2007).

In contrast to ERβ, ERα appears to be highly expressed by cancer cells and approximately 70% of breast cancer cases are diagnosed as ER-α positive (DeSantis et al., 2011). Unlike to ERβ, ERα seems to play a role in the onset (Hewitt et al., 2002) and progression of cancer (McPherson et al., 2008; Attia and Ederveen, 2012). Recently it has been shown that ERα dependent action inhibited P53/P21 expression and stimulated proliferative cell nuclear antigen (PCNA) and proliferation-related Ki-67 antigen expression in MCF-7 breast cancer cells (Liao et al., 2014). A low level of ERα expression was measured in normal human breast tissue but its expression was higher in some forms of breast cancer including invasive ductal carcinoma (Gustafsson and Warner, 2000) and in lobular cancer (Huang et al., 2014). ERα was consistently expressed in patients with non-small cell lung cancer where ERβ expression was variable (Kawai et al., 2005). However, in colon cancer ERα expression was reported to be similar to the surrounding normal tissue, and appeared to have no influence in colon carcinogenesis (Foley et al., 2000; Campbell-Thompson et al., 2001),

1.3.6. Estrogen receptors and obesity

There is evidence that both ERα and ERβ may have functions that protect against obesity. For example, with high fat feeding ERβ knockout mouse accumulated more lipid in gonadal adipose tissue compared with wild type. Effects of ERβ on lipid biogenesis may be mediated through PPARγ (Foryst-Ludwig *et al.*, 2008) which interacts with ERβ in a ligand-independent manner (Wang and Kilgore, 2002). In contrast, however, work in the

ovariectomized mouse indicated that ERβ has no role in diet-induced obesity (Roesch, 2006).

An ERα knockdown mouse model was obese and developed obesity related disorders including insulin resistance, glucose intolerance and lower energy expenditure compared with wild type (Heine *et al.*, 2000). Another study showed a reduction in the expression of ERα in obese female subjects compared with lean subjects (Nilsson *et al.*, 2007). In contrast to ERβ, ERα was found to be important in reducing food intake and preventing diet-induced obesity in ovacriectomized rats (Roesch, 2006).

1.3.7. The effect of estradiol/or genistein on estrogen receptor expression

It appears that the expression of ERs can be influenced by the presence of their activating ligands. 17β-Estradiol, the common endogenous mammalian ER ligand, was demonstrated to have differential effects on the expression of ERα versus ERβ. In vitro it was uncovered that 17β-estradiol treatment (1-20 nM) caused down-regulation of ERα in different cell models including M6 breast cancer cells (Yoshidome et al., 2000), MCF-7 breast cancer cells (Hall et al., 2008) and ZR-75-1 cells (Kousidou et al., 2008). However, ERβ expression was not affected by 17β-estradiol in SW480 intestinal cells (Hartman et al., 2009). Moreover, in sheep foetal primary endothelial cells 17β-estradiol (0.1-10 nM) was found to have time dependent effects on ER expression. Short exposure (2 h) to 17β-estradiol down regulated ER-α and had no effect on ERβ. However, a longer (6 h) exposure time caused ERα up-regulation and ERβ down-regulation (Ihionkhan et al., 2002).

As a ligand, genistein has a higher affinity toward ERβ than ERα (Zhu et al., 2006; Chrzan and Bradford, 2007). At the expression level it was shown that giving genistein to mice (50-200 mg/kg) downregulated both ERs in epididymal adipocytes (Penza et al., 2006). A low dose of genistein (1 μM) was found to have no effect on ERβ expression in MCF-7 cells but upregulated ERβ in T47D breast cancer cells. These differences were suggested to be related to the lower ERα/ERβ ratio in T47D compared with MCF-7 cells (Pons et al., 2014). In a study in 3T3-L1 and MCF-7 cells genistein (50-200 μM) was shown to down-regulate ERα dose and time dependently (Choi et al., 2014). Other phytoestrogens including tectorigenin (100 μM) upregulated ER-β in the LNCaP prostate cancer cell line (Stettner et al., 2007). Similarly, Belamcanda chinensis extract, which is a

mixture of around 13 different phytoestrogens, was also found to up-regulate ER-β in the same cell line (Thelen et al., 2007).

1.4. Chromatin

1.4.1. Chromatin structure

Humans have 46 chromosomes comprising approximately 6.5 billion base pairs of DNA. Each chromosome consists of a double stranded DNA molecule wrapped around sequential histone protein octamer cores. 145-147 base pairs of DNA coils around each histone protein octamer to form the basic unit of chromatin, the nucleosome (Mariño-Ramírez et al., 2005). The DNA between nucleosomes (linker-DNA) comprises 160-240 base pairs (McGhee and Felsenfeld, 1980). Linker histone proteins (histone H1) are located outside the nucleosome in association with the linker DNA (McBryant et al., 2010; Harshman et al., 2013). This structural arrangement of the chromatin is crucial for genomic stability and transcriptional control of the genome (Jiang and Pugh, 2009). This structural arrangement is shown schematically in Figure 11.

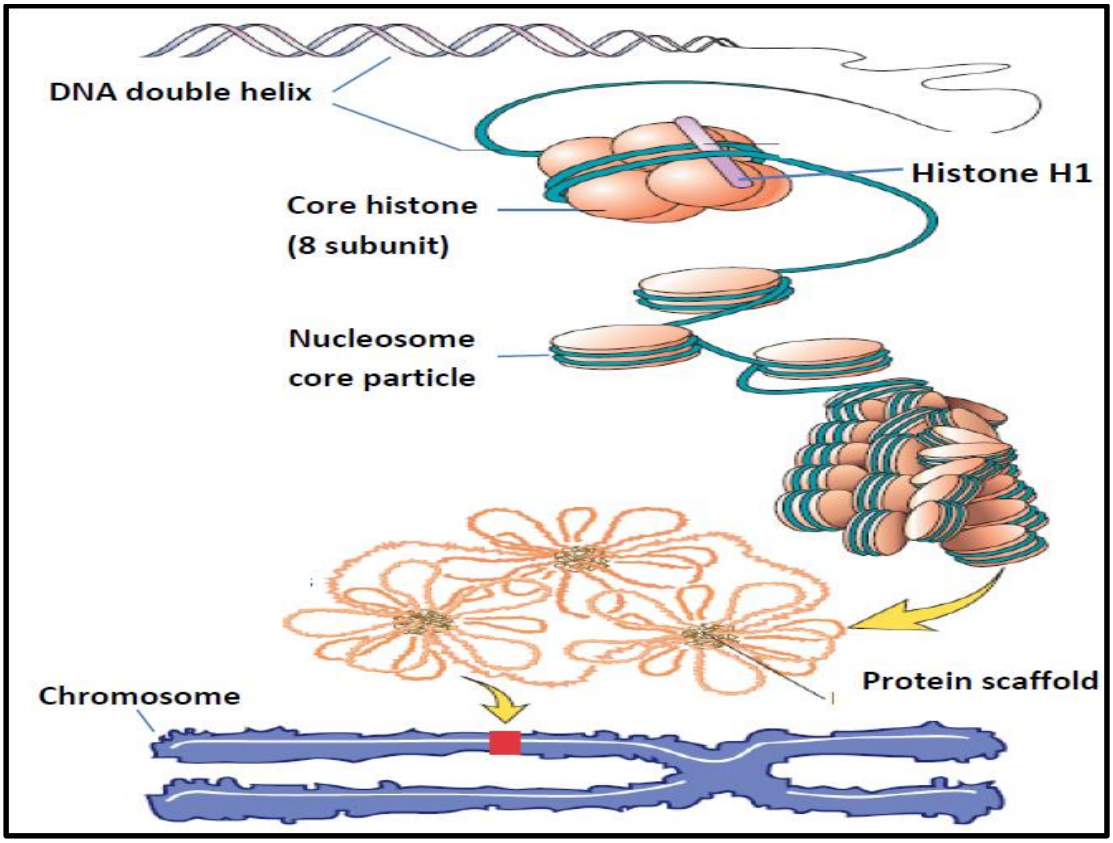
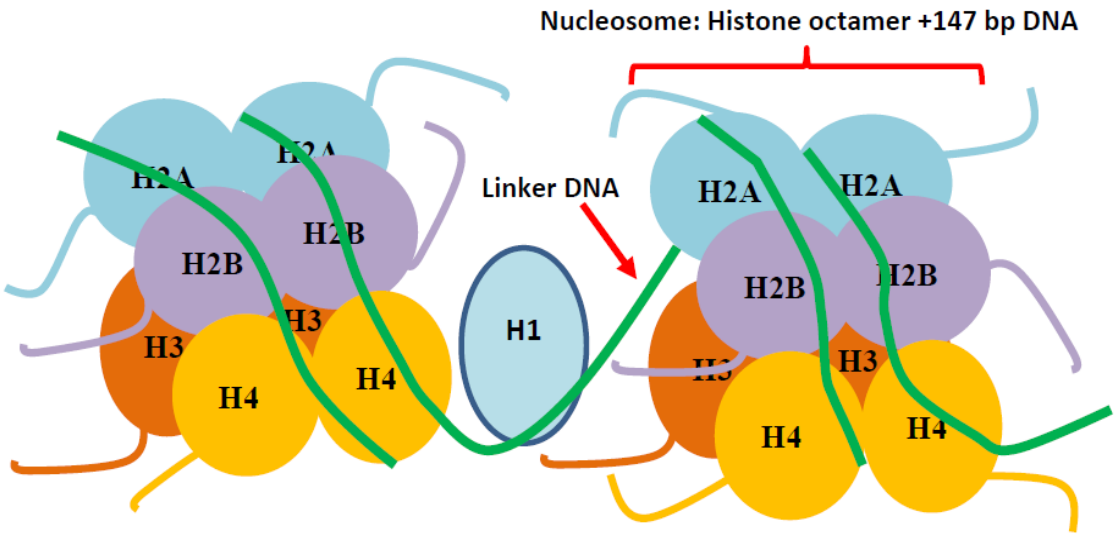


Figure 1. 11: Chromatin structure and arrangement of histone core proteins in association with the DNA and linker histone (Karp 2008).

1.4.2. Histone proteins

Histone proteins can be divided generally into core and linker histone proteins. The core histone proteins are highly organised and essential for nucleosome assembly. The core of each nucleosome is an octamer of histone proteins comprising two copies of each of H2A, H2B, H3 and H4 (Jiang and Pugh, 2009). The N-terminal regions of core histone proteins and the H2A C-terminal region extend out of the nucleosome and interact with other proteins via covalent modifications of these histone tails structures to regulate gene expression activity and other functions (Karp, 2008). Histone H1, the linker histone, does not participate in nucleosome formation but is bound to the linker DNA in close proximity to the nucleosome (Bustin et al., 2005). Figure 12 shows relationship between DNA and histones.



Histone	Number of residues	Mass (kDa)
H1	215	23.0
H2A	129	14.0
H2B	125	13.8
H3	135	15.3
H4	102	11.3

Figure 1. 12: Nucleosome structure consists of an octamer of histone proteins comprising two copies of each of H2A, H2B, H3 and H4 which is associated with linker DNA and histone H1. Histone proteins properties including the number of amino acid residues and mass is also shown.

1.4.3. Organization of histone genes

Cell division and proliferation requires the production of large quantities of histone proteins. This requirement is met by the presence of multiple copies of histone genes, which in higher eukaryotes cluster in tandem repeats or jumbled clusters of between 10 and 100 genes. In the human genome most histone gene clusters are on the short arm of chromosome 6. Other histone genes are scattered on chromosome 1, 4, 11, 17 and 22. There is a small cluster on chromosome 1 whereas the other chromosomes accommodate only single histone genes (Albig and Doenecke, 1997; Marzluff et al., 2002; Marzluff et al., 2008). The arrangement of the human histone genes is shown in Figure-13

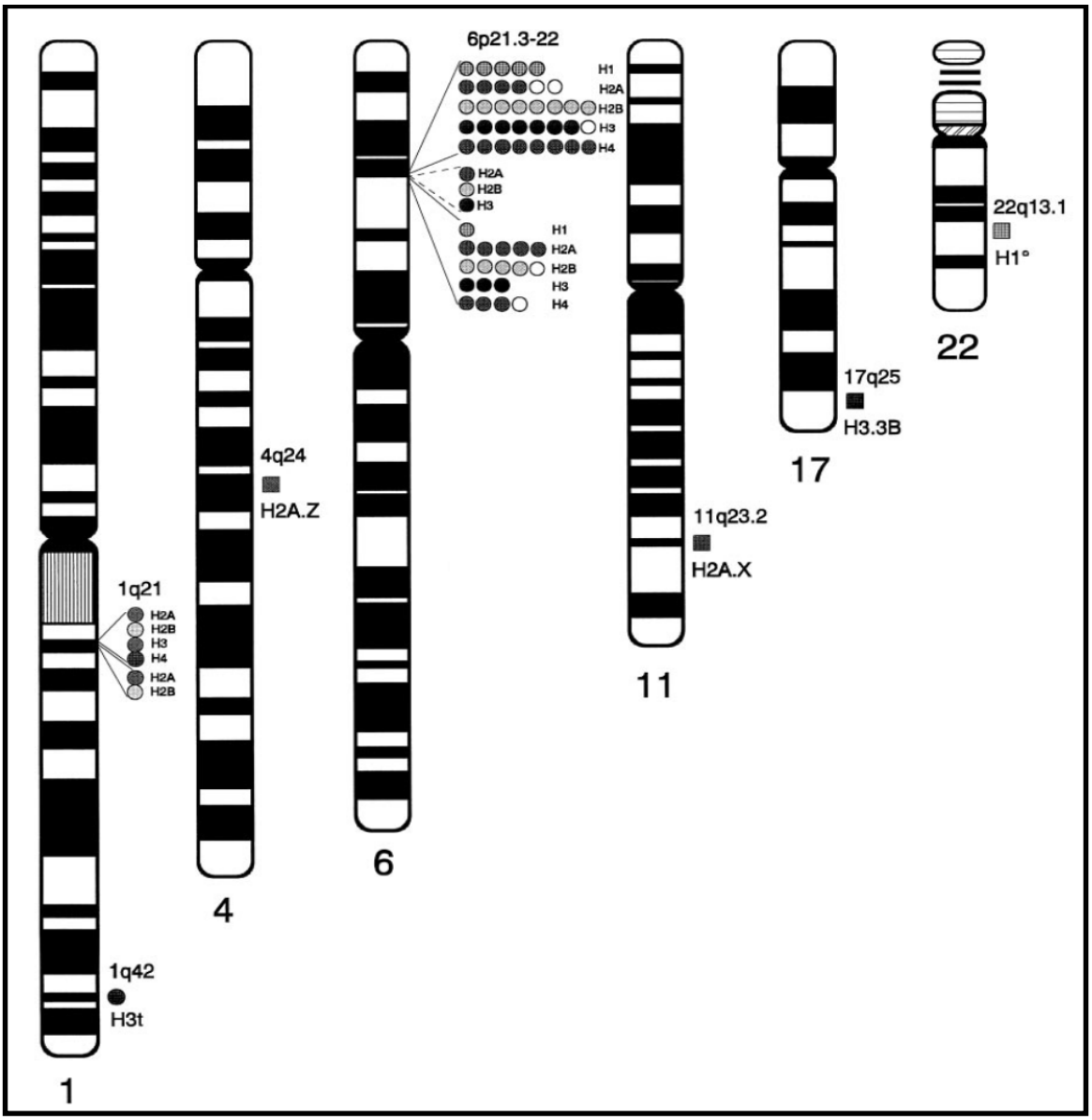


Figure 1. 13: Chromosomal distribution of the human histone genes (Albig and Doenecke, 1997)

1.4.4. Histone mRNA and protein synthesis

Approximately 90% of histone proteins synthesis occurs during S-phase of the cell cycle. This process is termed replication-dependent histone biogenesis. The remaining 10% is replication-independent and is the process through which canonical histone proteins are replaced and that keeps specific loci of the genome in the inactive heterochromatin or active euchromatin configuration state as required by cellular functional demands (Wu and Bonner, 1981; Chen et al., 2013a).

Specific proteins including stem-loop binding proteins (SLBP), the U7 small nuclear ribonucleic proteins (U7 snRNP) and heat labile factor participate in histone mRNA stabilization (Allard et al., 2005; Marzluff et al., 2008).

The mRNAs of the canonical replication-dependent histones in vertebrates are characterized by two main features that distinguish them from replication-independent histones: a hairpin-loop at the 3` end of the mature mRNA and lack of an intron (Marzluff et al., 2008). In addition to their translation into histone proteins, they require SLBP and stem loop binding protein interacting proteins 1 SLIP1 in order to interact with eukaryotic translation initiation factor-4γ (EIF4G) (Marzluff et al., 2008).

1.4.5. Histone variants

Histones H1, H2A and H3 have multiple variants, whereas in contrast to H2B and H4 show less variability (Kamakaka and Biggins, 2005). Specialised and distinct functions of histone variants are assumed to be through their interactions with different chromatin assembling factors which direct them to specific chromatin loci (Ahmad and Henikoff, 2002). Histone H3 variants appear to have vital roles in determining either euchromatin or heterochromatin formation. There are five main histone H3 variants in mammals, which comprise two replication-dependent (canonical) histones H3.1 and H3.2, and three non-canonical variants comprising a transcription-dependent histone variant (histone H3.3), the centromere specific histone variant (CENP-A) and testis specific histone H3 (H3t) (Loyola et al., 2006; Loyola and Almouzni, 2007; Szenker et al., 2011). There are very few differences in sequence especially between histone H3.1, H3.2 and H3.3 proteins (H3.1/H3.2, 99%, and H3.1/H3.3, 96%). In contrast, CENP-A is much more divergent and shares only 47% amino acid sequence identity with H3.1 (Loyola and Almouzni, 2007).

It was demonstrated that presence of histone H3.3 and/or its post-translational modifications, especially at specific gene promoter and enhancer regions, was associated with transcriptional activity (Ahmad and Henikoff, 2002; Hake and Allis, 2006; Chen et al., 2013a). Expression of this variant was found to be crucial for DNA replication during the S-phase of the cell division (Frey et al., 2014) and to be associated with the transcriptionally active sites of the genome in the chicken erythroid cell models HD24 and 6C2 (Jin and Felsenfeld, 2006). Abundance of this variant marks highly active and fully differentiated cells such as nerve cells (Das and Tyler, 2013). Histone H3.3 knockdown in mouse resulted in abnormal chromosomal segregation (Bush et al., 2013) and in defective decondensation of the pericentromeric chromatin (Lin *et al.*, 2013).

Histone H3.2 on the other hand appears to be associated preferentially with silenced loci and with heterochromatin formation, whereas histone H3.1 is likely to have bidirectional (activation and/or silencing) effects on transcriptional regulation (Hake and Allis, 2006). Replacement of histone H3.3 with histone H3.1 prevented C2C12 differentiation into myocytes and prevented lineage specific gene expression (Harada et al., 2014). Also histones H3.1 and H3.2 were found to be associated with heterochromatin formation during fertilization and embryogenesis in the mouse (Akiyama et al., 2011).

1.4.6. Histone modification

Another mechanisms through which histones control transcriptional activity is through their extensive post translational modifications (PTM), especially at the N-terminus (Strahl and Allis, 2000; Zhou et al., 2011). These modifications tend to be conserved among cell types and possibly among species (Roh et al., 2007; Ryba et al., 2010).

The most common histone modifications include acetylation, methylation, phosphorylation, poly ADP rybosylation and ubiquitination. These post translational modifications can cause either activation or repression of their associated genes (Martin and Zhang, 2007; Ruthenburg et al., 2007; Bannister and Kouzarides, 2011). For example H4k16 acetylation is generally associated with gene activation while H4K20 with inhibition (Nishioka *et al.*, 2002; Shogren-Knaak *et al.*, 2006).

Histone variants appear to be susceptible to specific forms of modification. For instance, histone H3.3 but not H3.1 was found to undergo extensive trimethylation at

lysine 4 and 27 (H3K4m3) which is a hallmark of active chromatin, and to be less susceptible to lysine 9 and 27 dimethylation (H3K9m2 and H3K27m2) which are associated with heterochromatin formation (McKittrick et al., 2004; Harada et al., 2014).

1.4.7. Histone proteins and lifespan

It has been posited that histone proteins and their posttranslational modification can be important in modulating life expectancy (Feser *et al.*, 2010b; Khan and Khan, 2010). There is a view supporting the idea that abundance of histone proteins can positively affect life expectancy (O'Sullivan et al., 2010). Substantiate evidences include that overexpression of histone proteins in yeast increased lifespan (Feser *et al.*, 2010b). It also reduced global histone protein expression and/or loss of histone proteins at specific regions of the genome correlated with genome instability and aging in yeast and in human foetal lung fibroblast cells (IMR90) (Dang et al., 2009; O'Sullivan et al., 2010; Hu et al., 2014). The expression of histone H3 and H4 was specifically lower in fibroblasts of old versus young human individuals, and at late passage (P75) compared with early passage (P30) of fibroblasts (O'Sullivan *et al.*, 2010). The ratio of histone variants including H2A and H3 variants was also found to be reduced compared with total histone expression level during aging in human lung embryonic fibroblast cells (Rogakou and Sekeri–Pataryas, 1999). In contrast to a reduction in histone protein abundance with age, histone protein level was found to be increased in aged versus young mice (Alatawi, 2012).

Histone modifications have also been correlated with life expectancy. The level of histone H3 lysine 9 tri-methylation (H3K9m3), histone H3 lysine 9 di-methylation (H3K9m2), and histone H3 lysine 56 acetylation (H3K56ac) decreased with age in human fibroblast cells and aged yeast (Dang *et al.*, 2009; O'Sullivan *et al.*, 2010). However, histone H4 lysine 16 acetylation (H4K16ac) increased in aged yeast (Dang et al., 2009).

1.4.8. Effects of genistein on histone expression

Histone protein expression can be affected by environmental and dietary factors (Ford et al., 2011; Dik et al., 2012; Suter et al., 2014). 1µM genistein or 0.1-2 µM 17β-estradiol treatment for 6 days was found to upregulate expression of the canonical histone proteins (H2A, H2B, H3 and H4) in MCF-7 cells but not in the ERα and/or ERβ negative cell lines MDA-MB-231, MCF-10F, MCF-10A (Zhu *et al.*, 2009). Another study in

MCF-7 cells showed that long term (40-60 day) genistein treatment (10 μM) reduced acetylated histone H3 expression (Jawaid et al., 2010).

1.5. DNA methylation

DNA methylation is the addition of a methyl group at Position C5 of cytosine where followed by a guanidine for example, at CpG sites. The DNA methylation status varies depending on cell phenotype and stage of differentiation (Morgan et al., 1999; Chong et al., 2007). The process is controlled by the DNA methyltransferase enzymes (DNMTs). DNMT1 maintains the DNA methylation pattern between daughter cells (Chong et al., 2007), whereas DNMT3a and DNMT3b establish de novo DNA methylation (Okano et al., 1999). DNA methylation status is very susceptible to modifications by environmental and dietary factors including stress and diet (Junien and Nathanielsz, 2007; Daxinger and Whitelaw, 2010; Ford et al., 2011; Uriarte et al., 2013). For example, maternal exposure to ethanol (Kaminen-Ahola et al., 2010) led to changes in in the coat colour of the agouti mouse as a result of changes in the DNA methylation at the methylation-sensitive promoter element that determines this phenotype (Morgan et al., 1999). In humans, folic acid (0.4 mg/day) supplementation for 10 weeks also increased DNA methylation in both colonic mucosa and leukocytes (Pufulete *et al.*, 2005). DNA methylation is considered to be an important likely contributor to the process of aging (Maegawa et al., 2010). Older individuals generally have lower global DNA methylation than younger individuals (Gomes *et al.*, 2012). DNA methylation also appears to be involved in the control of lipogenesis. For example, DNA methylation of leptin gene varies among different cell types and stages of cell differentiation (Stöger, 2006), being hypermethylated in preadipocytes then hypomethylated in mature adipocytes (Melzner et al., 2002). In mice *Ucp1* gene had higher DNA methylation in white adipocytes compared with brown adipocytes (Kiskinis et al., 2007). Furthermore, DNA methylation of the FAS and SREBP1 promoters was decreased in mice in response to a high caloric diet (Uriarte et al., 2013).

1.5.1. Effects of resveratrol on DNA methylation

Resveratrol was found to stimulate an increase in the DNA methylation of genes encoding pro-inflammatory cytokines including IL1β, IL-6, TNF-α and IFN-γ, associated with lower level of expression in rats (Lou et al., 2014). Effect of resveratrol on DNA

methylation is likely to be through affecting DNMT expression and activity (Paluszczak et al., 2010). In the support of this view, resveratrol was observed to down-regulate DNMT1 (Qin et al., 2005; Stefanska et al., 2012) and DNMT3b expression (Qin et al., 2005; Qin et al., 2014). Additionally,

Recently, it has been demonstrated that a high caloric diet can stimulate a reduction in the DNA methylation of genes involved in the process of lipogenesis including *Fasn* and *Srebp1* (Uriarte et al., 2013; Gracia et al., 2014). The dietary induced changes in the DNA methylation status, especially that of FAS, was prevented by pterostilbene (30 mg/kg/day), which is a dimethyl ether derivative of resveratrol. However, in spite of its effects to reduce diet-induced obesity in rats, resveratrol (30 mg/kg/day) was found to have no effect on FAS promoter DNA methylation, showing that antiobesogenic effects of resveratrol occur through mechanisms which are alternative or additional to DNA methylation (Gracia et al., 2014).

Aims and objectives

This study arose from previous work in the laboratory that had uncovered novel actions of resveratrol, specially that treatment of mouse 3T3-L1 adipocytes with resveratrol reduced mRNA for two key enzymes involved in lipogenesis – fatty acid synthase (FAS; *Fasn* gene) and acetyl Co-A carboxylase (ACC; *Acaca* gene)- and that resveratrol evoked a reduction in histone protein expression. The aim was initially to investigate these responses in more detail, in particular to determine details of the underlying molecular mechanism. A second major aim was to determine if genistein had similar actions, predicated on the fact that these two compounds are structurally similar. However, I discovered that actions of genistein were potentially of more interest and importance. In particular I observed that treatment of 3T3-L1 cells with genistein appeared to induce a beige adipocyte phenotype. Thus, the objectives of the wok evolved to be as follows

1. Determine if genistein affects the expression of genes that characterise both white (*Acaca, Fasn, Fabp4, Lipe, Rarres2, Retn*) and brown (*Ucp1, Tnfrsf9)* adipocytes, and/or affects the expression of genes that have been recognised as mediators of white to brown adipocyte interconversion (*Sirt1, Cebpb, Ppargc1a*) in 3T3-L1 cells.
2. Determine if genistein affects mitochondrial function in 3T3-L1 cells in a manner commensurate with a change in phenotype from white to beige adipocytes.
3. Determine if the effects of genistein that suggest conversion of white to beige adipocytes are a function of genistein binding to the ER.
4. Investigate the involvement of Sirt1 in the process of white into brown adipocyte interconversion.
5. Determine if genistein, like resveratrol, affects histone protein expression through effects on histone gene transcription and determine if such action is mediated through the ER.
6. Measure the effect of genistein on the expression of both ER subtypes, and compare this action with that of 17β-estradiol, based on evidence that expression of the ER can be auto-regulatory.
7. Investigate the effect of resveratrol on DNA methylation of *Acaca* and *Fasn* promoters, to address the hypothesis that this is the basis of the reduction in the corresponding mRNA species observed in 3T3L1 cells.

Chapter 2

2. Materials and Methods

2.1. Cell culture

Cell culture and preparation of media was carried out under aseptic conditions using a class-II laminar flow hood cabinet. Ethanol (70%) was used to clean all working area. Disposable plastic ware was purchased sterile. All reusable glassware and other equipment was sterilized by autoclaving.

2.1.1. 3T3-L1 Cells

3T3-L1 cells, which model adipocytes after differentiation under suitable conditions and were derived from disaggregated Swiss 3T3 mouse embryos (Green and Kehinde, 1975), were purchased from American Type Culture Collection (ATCC)].

2.1.1.1. Culture and differentiation of 3T3-L1 cells

Cryovials containing 3T3-L1 cells were removed from storage in liquid nitrogen and thawed in a waterbath (37°C/ 3-4 min). Cells were washed with 10 ml pre-warmed preadipocyte medium [Dulbecco's modified Eagle's medium containing Glutamax plus 4500mg/l glucose (Life Technologies), supplemented with 10% calf serum (Sigma), 1% penicillin/streptomycin]. Cells were collected by centrifugation at 100 g for 5 min. The supernatant fluid was discarded and the cell pellet was re-suspended in pre-warmed (37°C) preadipocyte medium. Cells were then seeded in 75 cm^2 flasks at 0.2 X 10^5/ml (Greiner Bio-one), then incubated at 37°C in 5% CO_2 in air.

Cells proliferation was monitored regularly after seeding using a phase contrast microscope (Olympus CK2) and cells were passaged when approximately become 70-80% confluent, after removing the spent medium, cells were washed with pre-warmed 1x Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $NaHPO_4$.7H2O, 1.4 mM KH_2PO_4, pH 7.3) (Sigma, D8538). Cells were detached from the flask by adding 2-3 ml of 0.05% trypsin plus 0.02 M EDTA (Sigma) and then incubating (37°C) for approximately 5 min. The detached cells were washed with 10 ml of pre-warmed preadipocyte medium and centrifuged (100 g for 5 min) to pellet the cells. Following re-suspension of the cell pellet in 10 ml of freezing medium (90% preadipocyte medium plus 10% DMSO) cells were aliquoted into cryovials (Corning) (1 ml/tube). Cells were held overnight at -80°C in a

freezing box (Nalgene®) containing isopropyl alcohol to reduce the temperature of the cells gradually then transferred into liquid nitrogen for long-term storage.

Fresh cells were used for each experiment. Thus all measurements were made on cells at the same passage number. Cells were passaged at the ratio of 1:10 in 75 cm^2 flasks and counted using a haemocytometer chamber slide (Neubauer) after mixing 15 µl of cell suspension with an equal volume of 0.4% Trypan blue dye solution (Sigma).

For differentiation, 3T3-L1 cells were grown in preadipocyte medium in 6-well plates (Griener). Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate by using preadipocyte medium supplemented with a differentiation cocktail containing 500 µM 3-isobutyl-1-methylxanthine (IBMX), 10µg/ml insulin (Sigma, I9278) and 250 nM dexmethasone (Sigma, D4902), 8 µg/ml biotin (Sigma, B4639) and 4 µg/ml pantothenic acid (Sigma, P5155). After a further 48 h, cells were fed with preadipocyte medium fortified with 10 µg/ml insulin, 8 µg/ml biotin and 4 µg/ml pantothenic acid and incubated for another 48 h. Thereafter, cells were maintained in mature adipocyte medium (0.2 µg/ml insulin, 8 µg/ml biotin and 4 µg/ml pantothenic acid), which was replaced every 2 days until the end of the experiment. Normally lipid droplets began to appear 5-6 days after inducing differentiation.

2.1.1.2. Staining 3T3-L1 cells with oil red-O

Oil Red O (Sigma, O1391, 0.5% w/v), which is a lipid specific stain, was diluted 3:2 in double distilled water to make up the working solution then sterilised by filtration through a sterile syringe filter (Biotech, 0.2µm pore size) after standing at room temperature for 30 min. Cells were washed with warm PBS, then fixed with 10% neutral formaldehyde for 2-3 h at room temperature then rinsed quickly with 60% isopropanol diluted in double distilled water and allowed to dry at the room temperature for approximately 10 min. cells were overlaid with freshly prepared Oil Red O and incubated for 2-3 h at room temperature. Cells were then washed 3-4 times with double distilled water and photographed under a light microscope.

2.1.2. Culture of Caco-2 and MCF-7 cells

2.1.2.1. Preparation of medium

Caco-2 complete medium was prepared by supplementing Dulbecco's modified Eagle's medium (DMEM, Life Technologies), which includes Glutamax plus 4.5 g/L glucose, with 10% v/v fetal bovine serum (FBS, Sigma), 60 µg/ml gentamycin (Sigma, G1397-10ml) and 1% v/v non-essential amino acids (NEAA) (Sigma, M7145).

MCF-7 complete medium was prepared by supplementing modified Eagle's medium (MEM, Sigma, M2279), with 2% Glutamax-100X (Life Technologies, 35050-038), 10% v/v fetal bovine serum (FBS, Sigma), 1% v/v non-essential amino acids (Sigma, M7145), 1% V/V sodium pyruvate (Life Technologies, 11360-039) and 60 µg/ml gentamycin (Sigma, G1397).

Phenol-free medium, for use when treating cells with phytoestrogens, was prepared by supplementing DMEM (Dulbecco's Modified Eagle Medium, Life Technologies), with 2% Glutamax (Life Technologies), 10% v/v fetal bovine serum (FBS, Sigma) and 1% sodium pyruvate (Life Technologies, 11360-039).

2.1.2.2. Passaging and seeding Caco-2 and MCF-7 cells

Routine culture of Caco-2 and MCF-7 cells was as described for 3T3-L1 cells (section, 2.1.1.1) but with the specific media described above. Caco-2 cells and MCF-7 cells were seeded at a density of 3.5×10^5 cells/ml - 4.5×10^5 cells/ml for experiments and cells were passaged at a 1:10 ratio for routine subculturing.

2.2. Treatment of cells with test substances

Stock solutions were prepared under sterile conditions and stored in aliquots at -20°C to avoid multiple freezing and thawing. Stock solutions of 100 mM resveratrol (Sigma, R5010), 100 mM genistein (Sigma, G6776), 0.5 mM fulvestrant (Sigma, 14409), 50 mM EX-527 (Sigma, E7034) and 10 mM TTNPB (Sigma, T3757), were prepared by dissolving them separately in dimethyl sulfoxide (DMSO, Sigma, D4540), while 1 µM β-estradiol (Sigma, E4389) was prepared in double distilled water. Stock solutions were added in the required volumes to the cell culture medium and the same volume of solvent only was added to control medium to ensure that DMSO concentration was constant under all experimental conditions. Solutions were then filtered through a sterile filter

syringe (Biotech, 0.2 µm pore size). Cells were treated for specific periods of time with test substances, which were added to the medium and replaced every 2 days until the end of the experiment, and at specific times after seeding and/or transfection as specified in relation to particular experiments. There was no decline in RNA yield with increasing concentration of genistein indicating that there were no toxic effects (table 1).

2.3. Primer design

Oligonucleotide primers were designed manually based on mRNA sequences as identified through NCBI (http://www.ncbi.nlm.nih.gov/). Primers were designed to be 18-25 nucleotide long with CG content 50-60% and where possible with melting temperature greater than 55°C. Sequences were assessed to check for chance of hairpin/self-dimerization or self-complementary using online software (http://www.basic.northwestern.edu/biotools/OligoCalc.html). Primers were synthesized by Eurofins MWG.

2.4. RNA measurement

2.4.1. RNA extraction

Cells were washed with 1 x PBS (1 ml/well), then TRIzol® Reagent (Life Technologies, 15596018) was added (1ml/well) and the mixture was then collected in a microfuge tube then stored at -80C°. Total RNA was then extracted using the PureLink™ RNA Mini Kit (Life Technologies, 12183018A), following the manufacturer's protocol. Briefly, 0.2 ml chloroform was added to the TRIzol cell preparation, then the mixture was shaken vigorously for 15 s then incubated for 2-3 min at room temperature then centrifuged (12000 g for 15 min at 4°C) to separate the cell components. The clear upper phase containing RNA (approximately 0.4 ml) was transferred into a sterile microfuge tube, an equal volume of 70% ethanol was added and the reagents were combined by pulse vortexing then transferred to the spin-cartridge, centrifuged (12000 g/15 s at room temperature) and the flow through was discarded. The cartridge was then washed with 0.7 ml and 0.5 ml wash buffer I and II respectively. After drying the membrane by centrifuging at 12000 g for 1 min at room temperature, RNA was eluted using 30 µl RNase free water as provided in the kit. Concentration was measured using a Nanodrop spectrophotometer and RNA was stored at -80°C.

2.4.2. Measurement of RNA and DNA concentration

RNA and DNA concentrations were measured routinely using a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific). Absorbance ratios 260/280 nm and 260/230 nm of 1.8-2.2 were considered to indicate acceptably pure preparations. RNA integrity was not measured (eg. By measuring RIN value) because the cost would be prohibitively high for the large number of samples analysed. However, the same procedure for preparing RNA for analysis by hybridization to DNA measurements carried out in the laboratory yielded RNA with RIN values of >7 (Ions, 2011). Moreover RNA integrity is much smaller potential confounding factor for the measurement of specific mRNAs by RT-qPCR to amplify short (<200 bp) region than for analysis using DNA microarray.

RNA yield			
Treatments	**Conc. ng/µl**	**260/230**	**260/280**
Control	364.6	2.07	1.91
Control	368.8	2.07	1.85
Control	295.4	1.98	2.15
10 µM genistein	499.4	1.96	2.08
10 µM genistein	526.4	2.07	2.01
10 µM genistein	455.8	2.07	1.86
50 µM genistein	590.3	2.02	2.03
50 µM genistein	436	2.01	2.07
50 µM genistein	407.1	2.06	2.08
100 µM genistein	392.8	2.08	2.04
100 µM genistein	308.8	2.09	2.04
100 µM genistein	360.1	2.07	1.87

Table 1: an example of the RNA concentrations obtained from 3T3-L1 cells treated with different concentrations of genistein.

2.4.3. Reverse transcription of RNA to cDNA

RNA (0.5-1 µg) from cells (3T3-L1, Caco-2 cells or MCF-7) was reverse transcribed to cDNA using Superscript™ III RNase H- Reverse Transcriptase (Life Technologies) in two steps. First, RNA (approximately 1 µg) was added to 0.4 µl of 0.5 mM dNTPs (Bioline), 1 µl random primers (150 ng/µl; Promega) and RNase free water in a final volume of 13 µl. The reaction was held at 65 ºC for 5 min then samples were transferred onto ice. Second, 1 µl

RNase Inhibitor (40 U/µl; Promega), 1 µl Superscript™ III RT (200 U/µl), 4 µl 5x first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl2) and 1 µl DTT (0.1 M) were mixed into the reaction by pipetting. The reaction was then heated to 25 ºC for 5 min, 50 ºC for 45 min, and then 70 ºC for 15 min using a Thermohybaid PX2™ thermal cycler. Samples prepared in an identical manner but omitting the addition of Superscript™ III were used as negative controls in downstream PCR to detect/ensure absence of DNA contamination.

2.4.4. RT-qPCR

The relative quantification method was used for RT-qPCR: Reactions were set up in LightCycler® 96 well plates (Starlab, I1402-9909), to contain 1 µl cDNA (RTR-reaction), 5 µl SYBR Green I Master Mix (Roche), 1 µl (0.5 µM) sense and antisense primers (Eurofins MWG; Tables 1 and 2) made up to total of 10 µl using sterile nucleic acid free water (Roche). Cycling parameters were 95˚C/5 min initial denaturation, 95˚C/10 s denaturation, followed by 45-55 cycles of 55-60˚C/10 s annealing, 72˚C/15 s elongation.

Each sample was measured in duplicate. Fluorescence was detected for each PCR cycle and the threshold crossing points (Ct values) determined. At the end of all reactions a melting curve was generated to ensure the specificity of target gene amplification.

Expression of all target and reference genes were calculated for each sample using $2^{\Delta\Delta Ct}$. Each target cDNA concentration was then expressed in relation to the average value of the reference gene from the same cDNA preparation. Two reference genes were used for all measurements.

2.5. Agarose gel electrophoresis

Agarose gels (1% or 2%) were prepared by boiling 0.5 or 1 g agarose in 50 ml of 1 x TBE buffer (Tris base 10.8 g, Boric Acid 5.5 g, EDTA 0.93 g, 1 L double distilled water pH 8.3). Before pouring the gel, 2 µl of Safe View nucleic acid stain (NBS Biologicals Ltd, NBS-SV) was added. Samples were mixed with loading dye (50 mM Tris-HCl pH 8, 5 mM EDTA, 20 % glycerol and 0.1 % Bromophenol Blue) in a ratio of 1:5 (1 µl of the dye and 5 µl of sample), and loaded onto the gel in parallel with a molecular weight marker mixture (Ladder I-V, Bioline). Gels were run at 60 V for 50 min then DNA bands were visualised using a UV transilluminator (Uvitec BTS-26M).

2.6. Measurement of mitochondrial function

2.6.1. Cell seeding on Seahorse V7 cell plates

Mitochondrial function was measured using the Seahorse XF-24 analyser (Seahorse Bioscience), 3T3-L1 cells were cultured in Seahorse V7 cell plates (Seahorse Bioscience). Cells were induced to differentiate and maintained to grow in the presence and absence of genistein as described in section 2.1.1.2.

2.6.2. Pre-test plate preparation

The Seahorse V7 cell plate cartridge was hydrated with Seahorse calibration buffer (Seahorse Bioscience) and incubated without CO_2 at 37°C for approximately 4-5 h before use. Solutions for injection were prepared from stock solutions and assay medium, then loaded into the injection ports. Briefly, 50 µl of oligomycin (10 µg/ml) (Sigma, O4876) was added to port A, 55 µl TTNPN (10 µM) (Sigma, T3757) was added to port B, 60 µl of FCCP (2.5 µM) (Sigma, C2920) was added to port C, 65 µl antimycin A (2.5 µM) (Sigma, A8674) was added into port D. After loading solutions the cartridge was loaded into the Seahorse XF-24 analyser for calibration.

2.6.3. Preparation of the assay medium

On the day of the assay, 35.l ml of assay medium was prepared from 33.6 ml of pre-warmed DMEM (Sigma, D5030) by adding 31.5 mg D-glucose (Sigma, G8270), 2% L-Glutamax (Life Technologies), 3% calf serum (Sigma, C8056-500ML), 1% sodium pyruvate (Life Technologies).

2.6.4. Washing and replacing cell culture medium with the assay medium

Carefully and gently 400 µl of the cell culture medium (from a total of 450 µl) was removed and replaced with assay medium (400 µl), a further volume of 400 µl/well was then replaced again with another 450 µl/well, giving a final volume of 500 µl per a well. The plate was incubated in the absence of CO_2 at 37°C for 1 h before use. Finally the plate was installed into XF-24 analyser to measure mitochondrial function. All data were expressed relative to total protein (section; 2.9.2), which was measured by preparing cell lysate at the end of the experiment.

2.7. Generation and manipulation of promoter-reporter plasmid DNA constructs

2.7.1. Identifying gene promoter sequences

The region 1000-1500 base pairs upstream to the transcription start site was taken as the promoter region for each gene. Sequences were retrieved in FASTA format by searching NCBI (http://www.ncbi.nlm.nih.gov/). Promoter sequences identified in this way were confirmed by alignment against the transcript sequence using Clustal software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.7.2. Amplification of DNA products by PCR

Required region of DNA were isolated and amplified by PCR. Reactions of 50 µl were prepared by mixing 25 µl of HotStart Taq master mix (Qiagen), 1 µl (10 µM) each of sense and antisense primers (Table 3), approximately 50-200 ng of template (the volume dependent on the concentration and type of the template) and double distilled water to make up the volume. To check for DNA contamination negative control reactions were prepared by adding water instead of the template. Generally the following PCR program was used:

95°C for 15 min (to activate Hot Start polymerase)

35 Cycles
- 95°C for 30 s denaturation
- 50-60°C for 30 s annealing temperature
- 72°C for 1 min elongation

72°C for 10 min final elongation

4°C holding temperature

For subcloning PCR products were analysed by agarose gel electrophoresis to confirm successful amplification then purified using the Qiamp PCR purification kit (Qiagen).

2.7.3. Digestion of DNA with restriction enzymes

Plasmid vector and DNA inserts (PCR products) were digested by mixing 1µl (10u/µl) *Hind* III (Promega, R604A), 1µl (12u/µl) *EcoR*1 (Promega, R601A), 2.5 µl buffer E (Promega, R005A), 0.25 µl of acetylated bovine serum albumin (BSA; Promega, R396E), 500-1000 ng template in a total volume 25 µl, made up with double distilled water.

Reactions were incubated at 37 °C for 1-2 h. Products were resolved by agarose gel electrophoresis then purified by using the Qiaquick PCR purification kit (Qiagen, 28104).

2.7.4. Subcloning PCR product into pSF-pA-PromMCS-BetaGal

For subcloning into pSF-pA-PromMCS-BetaGal (Oxford genetics, OG239) ligation reactions comprised 30-60 ng vector with 90-180 ng DNA (1:3 molar ratio), 1 µl 10X T4 DNA ligase buffer (Promega, C126B), 4 µl double distilled water and 1 µl (1-3u/µl) T4 DNA ligase (Promega, M180A). The reaction was left to stand at 4°C/overnight, then used for bacterial transformation.

2.7.5. Bacterial transformation and preparation of plasmid DNA

Approximately 5 µl of ligation reaction was added to competent DH5α *E. coli* cells (Invitrogen) in a microfuge tube. After mixing gently the tube was incubated on ice for 30 min, then cells were heat-shocked at 42°C for 45 s, and incubated on ice for further 5 min. After addition of 250 µl of Luria Bertani (LB) medium (1% peptone, 0.5% yeast extract and 1% NaCl) cells were incubated for 1-2 h in a shaking incubator at 37°C/250 rpm, then the cells were spread on LB agar plates that contained either 100 µg/ml ampicillin or 50 µg/ml kanamycin, depending on the plasmid vector, and incubated at 37°C overnight. A starter culture (LB containing ampicillin or kanamycin) from a well isolated colony was grown for 6-7 h in a shaking incubator (37°C/250 rpm) then used to inoculate 200 ml of LB medium containing 100 µg/ml ampicillin or 50 µg/ml kanamycin at a ratio of 1:100. The culture was grown overnight in a shaking incubator at 37°C/250 rpm. Finally, cells were harvested by centrifugation at 5000 g for 15 min at 4°C and DNA was extracted using the Endotoxin-free plasmid maxiprep kit (Qiagen) following the manufacturer`s protocol.

2.7.6. Preparation of plasmid DNA for sequencing

A well isolated colony was selected and grown in LB containing 100 µg/ml ampicillin or 50 µg/ml kanamycin for 14-16 h in a shaking incubator (37C°/250 rpm). The Qiaprep® Spin Miniprep-Kit (Qiagen, 27104) was used to prepare plasmid DNA, following the manufacturer`s protocol.

2.8. Transient transfection of mammalian cells and reporter gene assay

2.8.1. Transient transfection

Before transfection, cells were seeded (3.5×10^5 cells/ml) in six-well plates (Greiner) in complete medium. Twenty four hours later when the cells reached 50-60% confluence, cells were transfected with plasmid constructs. Briefly, for each well, 4 µl GeneJammer transfection reagent (Agilent technologies, 204130) was mixed with 100 µl of Opti-MEM medium (with no additions) (Invitrogen, 51985-026) and the mixture was incubated at room temperature for 5 min before adding 1.7-2 µg of plasmid DNA. This mixture was incubated at room temperature for further 15-20 min. The mixture was prepared in one single volume, scaled up as appropriate depending on the number of wells for transfection. During this time, spent medium was replaced with 900 µl of fresh phenol free medium in six-well plates then 100 µl of the transfection mixture was added drop-wise with gentle swirling. Cells were then incubated at 37°C/5% CO_2 for 4 h then wells were topped up with 500 µl phenol free medium. Cells were treated with test compounds 24 h after transfection.

2.8.2. Whole cell lysate preparation

After removing medium cells were washed with cold 1X-PBS (1ml/well) (Sigma) then 200 µl of cold lysis buffer (0.25 M Tris-HCl (pH 7.4), 0.25% (v/v) NP-40, 2.5 mM EDTA) was added to each well. Cells were then frozen at -20°C for 30 min then thawed at room temperature and the cell lysates were transferred into microfuge tubes using a sterile disposable cell scraper (Fisherbrand® Disposable Cell Lifters, Fisher Scientific). Cell lysates were then centrifuged at 12000 g for 5 min at 4°C. Supernatant fluids were transferred into new clean microfuge tubes, kept on ice and used on the same day for reporter gene assay by measuring beta-galactosidase activity.

2.8.3. Measurement of protein concentration

Protein concentration was measured using the Bradford assay. Briefly, protein samples were diluted 1:50, and standards were prepared using 0.1 mg/ml BSA serum albumin (BSA) which was diluted to give standards ranging from 0 to 0.1 mg/ml. Two hundred microliters protein solution was mixed with 800 µl of Bradford reagent (Bio-Rad), which was diluted 1:5 with double distilled water then volume of 250 µl were loaded in

triplicate into 96 well microplate. Absorbance at 595 nm was measured using a plate reader (ThermoLab Systems Multiscan Acent).

2.8.4. Measurement of Beta-galactosidase enzyme activity

β-galactosidase activities were determined based on a colorimetric assay involving the hydrolysis of the substrate chlorophenol red–β-D-galactopyranoside (CPRG) to release chlorophenol red. Twenty microliters of cell lysate was mixed with 130 µl of substrate (1.2 mg/ml chlorophenol red-β-D-galactopyranoside (CRPG, Sigma), which was diluted in buffer A (25 mM MOPS, 100 mM NaCl, 10 mM MgCl2, pH 7.5), in 96-well plates. The plate then was incubated at 37°C and the reactions were allowed to progress until the colour had changed from yellow to red. The reaction was stopped by adding 80 µl of stop solution (0.5 M Na_2CO_3) and the incubation time was recorded. β-galactosidase specific activities were calculated as nmoles of chlorophenol red formed per min per mg of protein (nmoles/min/mg).

2.9. Western blot analysis

2.9.1. Antibodies

Primary antibodies used were anti-histone H2B (Abcam, ab1790; 1:10000), Anti-histone H3 (Abcam, H0164; 1:5000), anti-histone H4 (Abcam, ab10158; 1:1000), anti-α tubulin (Sigma, T6074; 1:2000) and anti-β-actin (Santa cruz, SC-1615; 1:5000). Infra-red labelled secondary anti-bodies were used to visualise protein bands and were either donkey anti-Rabbit IgG (Li-Cor, 926-32223; 1:1000) or goat anti-mouse IgG (Li-Cor, 926-32220; 1:1000). All antibodies were diluted in a solution prepared by combining wash solution (0.1% tween-20 plus 1X PBS) with Odyssey blocking buffer (Li-Cor, 927-40000) in a 1:1 ratio.

2.9.2. Preparation of cell lysates

Cells were re-suspended using a sterile cell scraper in a mixture of 1X PBS and protease inhibitor cocktail (Roche, 11836153001) (1 ml/well) which was prepared by dissolving 1 tablet of protease inhibitor cocktail tablet in 10 ml PBS. Cells were placed in microfuge tubes and centrifuged at 13000 g for 15 min at 4C°. The supernatant fluid was discarded and cell pellets were resuspended in 50 µl of RIPA lysis buffer (Sigma, R0278-50ml) plus protease inhibitor cocktail then frozen at -80°C. Lysates were thawed and then

centrifuged at 13000 g for 20 min to remove cell debris. The supernatant fluid was transferred into new sterile microfuge tubes. Protein concentration was measured using the Bradford assay as described in section 2.8.3.

2.9.3. Preparation of SDS polyacrylamide gels

Separating gel (12.5%) was prepared using 2.19 ml 40% bis acrylamide (37.5:1), 2.8 ml 2.5X separating gel buffer (1.87 M Tris-HCl pH 8.9, 0.25 % SDS), 6 µl of TEMED, 65 µl of 10% ammonium persulfate (APS) in a total volume of 7 ml. Stacking gel (10%) was prepared using 0.25 ml 40% bis acrylamide (37.5:1), 0.4 ml 5X stacking gel buffer (0.3 M Tris-HCl pH 6.7, 0.5% SDS), 2.5 µl TEMED, 18 µl 10% APS in a total volume of 2 ml. The separating gel was poured between assembled glass plates (Bio-Rad) and overlaid with 2 ml of isopropanol to remove air then left for approximately 25 min to polymerise. Isopropanol overlaid was removed then stacking gel was added, inserting the comb to create wells. The gel was allowed to stand for further 20 min before use.

2.9.4. Preparation and electrophoresis of protein samples

Protein samples (5-10 µg) were mixed with protein loading buffer (45 mM Tris-HCl pH 6.8, 10% glycerol, 1% SDS, 0.01% bromophenol blue, 50 mM DTT, and 5% β-mercapto-ethanol), and heated at 95°C for 4-6 min then loaded onto acrylamide gels in parallel with a molecular weight marker (ColorBurst Marker MW 8-220 kDa, (Sigma). Electrophoresis was at 100-120 V for 1-2 h in 1X protein electrophoresis running buffer (0.5 M Tris-HCl pH 8.8, 1.9 M glycine, 0.1% SDS.

2.9.5. Transfer of protein onto PVDF membrane

Proteins were transferred from gels onto PVDF membrane (Hybond P, Amersham) by semi-dry transfer using an electroblotting cassette (TE22, GE Life Science). Briefly, a PVDF membrane was pretreated with 100% methanol for 30 s. Membranes were then rinsed with double distilled water for 2 min on a shaker at room temperature. Membranes were then incubated in transfer buffer (0.04 Tris-HCl pH 8.8, 1.5 M glycine, 0.08% SDS, 20% methanol) for 10-12 min at room temperature. Gels and membranes were then placed in contact and sandwiched in cassette between Whatman paper blotting paper. Transfer was at 15V for 50 min.

2.9.6. Probing membranes with antibodies

Membranes were cut into two pieces before being probed with primary antibodies against target (one half of the membrane) or reference proteins (other half). Membranes were allowed to air dry completely and incubated in membrane blocking solution (1 volume of Odyssey Blocking Buffer plus 1 volume of 1X-PBS) for one hour at room temperature with shaking. After blocking, membranes were incubated with primary antibodies for 1 h at room temperature with gentle shaking. Following washing (4 x 15 min) with membrane washing solution (0.1% Tween-20 in 1X-PBS), infra-red labelled secondary antibodies, protected from direct exposure to light, were applied for another hour on a shaker at room temperature, then membranes were washed again (4 x 15 min) with membrane washing solution. Membranes were rinsed with 1X-PBS and scanned using the Odyssey infra-red imaging system (LI-COR). Odyssey densitometry software was used to quantify protein bands.

2.10.Measurement of DNA methylation

2.10.1. DNA extraction

After removing spent medium, cells were washed and collected into pre-warmed 1X-PBS (1ml/well) in microfuge tubes using a disposable cell scraper. Genomic DNA was extracted from cells using the QIAamp® DNA Mini Kit (Qiagen, 51306) following the manufacturer`s protocol. Briefly, the cell suspension was centrifuged at 500 g for 5 min and the cell pellet was re-suspended in 200 µl 1X-PBS then 20 µl of proteinase K (Qiagen) and 200 µl of buffer AL (Qiagen) were added, and the reagents were mixed by pulse-vortexing for 15 s. Following incubation at 56°C for 10 min and brief centrifugation, 200 µl ethanol (100%) was added. After thorough mixing samples were applied onto mini-spin columns and centrifuged for 1 min at 6000 g. After washing with buffers AW1 then AW2 (Qiagen) the spin column was dried by high speed centrifugation (10000 g/1min). DNA was eluted into DNase-free tubes using double distilled water.

2.10.2. Bisulfite conversion of DNA

200-500 ng of genomic DNA was bisulfite modified using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corp, D5006) following the manufacturer’s protocol to convert non-methylated cytosine to uracil. Briefly, 130 µl of CT conversion reagent (900 µl double

distilled water, 300 µl M-Dilution Buffer, and 50 µl M-Dissolving Buffer) was mixed with 20 µl of DNA in a 0.5 µl microfuge tube. The samples were then incubated at 98°C for 10 min then 64°C for 2.5h. M-Binding Buffer (600 µl) was added to each sample then samples were applied to Zymo-Spin™ IC Columns. Columns were then washed with 100 µl M-Wash Buffer (with centrifugation at 10000 g for 30 s). M-Desulphonation Buffer was then added (200 µl per tube) and incubated with sample at room temperature for 15-20 min. Samples were centrifuged again (10000 g for 30 s) then columns were washed twice with 200 µl of M-Wash Buffer and DNA was eluted into DNase-free tubes using 10 µl of M-Elution Buffer with centrifugation (10000 g/30 s).

4 µl of the bisulfite modified DNA was used in a PCR reaction (see section 2.7.2) to amplify the required region using specific biotinylated primers (Table 4) then run on an agarose gel (section 2.5) to confirm successful amplification.

2.10.3. Pyrosequencing using the Qiagen PyroMark MD system

Pyrosequencing was carried out using biotinylated PCR product (section 2.10.2). The biotinylated DNA strand was separated then used as template in the pyrosequencing reaction. Briefly, 10 µl of biotinylated DNA was incubated with 80 µl binding master mix (containing 2 µl streptavidin-coated sepharose beads (GE Healthcare), 38 µl binding buffer (Qiagen) and 40 µl H_2O) on a shaker for 10 min. At the same time 12 µl sequencing primer (diluted in annealing buffer; Qiagen) was added into each well of the Pyromark MD 96 well plate (Qiagen). The PyroMark Vacuum Prep Workstation vacuum tool (Qiagen) was prepared in double distilled water for 25 s and then the biotinylated PCR product/binding master mix was applied. Sequential washing followed (5 s/each solution) using 70 % ethanol, 0.2 mol/L NaOH solution (denaturing buffer) and wash buffer (Qiagen). The vacuum pump was switched off, and the vacuum pump probes were submerged into the Pyromark MS 96 well plate containing sequencing primer which led to release of the biotin tagged, single stranded DNA into the wells. The plate was then incubated at 80ºC for 2 min, cooled to room temperature and transferred to the PyroMark MD™ system (Qiagen). The principle of pyrosequencing depends on detecting pyrophosphate (PPi) released as a result of deoxribonucleotide triphosphate (dNTP) incorporation into the complementary DNA strand. The released PPi is used by ATP sulfurylase to synthesise ATP in the presence of adenosine 5 phosphosulfate (APS). The ATP generated is used for luciferase-mediated

conversion of luciferin into oxylucifirin, which is accompanied by emission of light. The amount and the intensity of the generated light is proportional with the quantity of ATP. Unincorporated ATP and nucleotides are degraded by apyrase, which is a nucleotide degrading enzyme (Qiagen).

2.11.Statistical analysis

SPSS software (one way ANOVA and Student`s unpaired T-test) was used for statistical analysis. Data are presented as mean ± standard error (SE) or standard deviations (SD). $P \leq 0.05$ was considered statistically significant.

Table 2: Oligonucleotide sequences and thermal cycling parameters used for RT-q PCR in samples from *Mus musculus*. Sequences are in the 5'-3' direction as indicated below. Times are given in the order denaturing, annealing and extension.

Gene product	Purpose	Transcript Genbank accession number	Primer sequence (5'---> 3')	Anneal temp. (^{o}C)	Cycling parameters	No. of cycles
FAS	RT-qPCR	NM_007988.3	**Forward primer**: GTGGACATGGTCACAGATG **Reverse primer:** CATAGCTGACTTCCAACAGC	55	95^{o}C, 10 s 55^{o}C, 5 s 72^{o}C, 10 s	45
ACC	RT-qPCR	NM_133360.2	**Forward primer:** ATGTCCTGGATAACCTGGTC **Reverse primer:** GATATCCTGCAGCTCTAGCA	55	95^{o}C, 10 s 55^{o}C, 5 s 72^{o}C, 10 s	45
AP2	RT-qPCR	NM_024406.2	**Forward primer:** GACAGGAAGGTGAAGAGCAT **Reverse primer:** GTGGAATGTGTTATGAAAGGC	55	95^{o}C, 10 s 55^{o}C, 5 s 72^{o}C, 10 s	45
HSL	RT-qPCR	NM_010719.5	**Forward primer:** GTCAGTGCCTATTCAGGGAC **Reverse primer:** AGTTGAGCCATGAGGAGGC	55	95^{o}C, 10 s 55^{o}C, 5 s 72^{o}C, 10 s	45
Chemerin	RT-qPCR	NM_027852.2	**Forward primer:** CTGTGCAGTTGGCCTTCCAAG **Reverse primer:** GGTTTGATTGTGCACTCCGG	55	95^{o}C, 10 s 55^{o}C, 5 s 72^{o}C, 10 s	45

Resistin	RT-qPCR	NM_022984.4	**Forward primer:** CAGAACTGAGTTGTGTCCTGC **Reverse primer:** CTTGTCGATGGCTTCATCGATG	55	95°C, 10 s 56°C, 5 s 72°C, 10 s	45
Sirt1	RT-qPCR	NM_001159589	**Forward primer:** GCTGTGAAGTTACTGCAGG **Reverse primer:** GCAAGGCGAGCATAGATAC	56	95°C, 10 s 56°C, 5 s 72°C, 10 s	45
C/EBPβ	RT-qPCR	NM_009883.3	**Forward primer:** CAAGGCCAAGATGCGCAAC **Reverse primer:** GCAGCTGCTTGAACAAGTTC	56	95°C, 10 s 56°C, 5 s 72°C, 10 s	45
UCP1	RT-qPCR	NM_009463.3	**Forward primer:** CCTGCCTCTCTCGGAAACAA **Reverse primer:** TGTAGGCTGCCCAATGAACA	60	95°C, 10 s 60°C, 5 s 72°C, 10 s	55
CD-137	RT-qPCR	NM_011612.2 NM_001077508.1 NM_001077509.1	**Forward primer:** TCATTGTGCTGCTGCTAGTGG **Reverse primer:** CTGCACACACTCTGCAGATGT	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
PGC1α	RT-qPCR	NR_027710.1 NM_008904.2	**Forward primer:** CAATTGAAGAGCGCCGTGTG **Reverse primer:** GTCACAGGTGTAACGGTAGGTG	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
ERα	RT-qPCR	NM_007956.4	**Forward primer:(Wang *et al.*, 2012)** TGGGCTTATTGACCAACCTAGCA **Reverse primer:** AGAATCTCCAGCCAGGCACAC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45

ERβ	RT-qPCR	NM_207707.1 NM_010157.3 NR_104386.1	**Forward primer: (Wang *et al.*, 2012)** GACTGTAGAACGGTGTGGTCATCAA **Reverse primer:** CCTGTGAGGTAGGAATGCGAAAC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3 (H3.1, H3.2, H3.3)	RT-qPCR	NM_178203.1 NM_178216.3 NM_178205.2 NM_178204.2 NM_013548.4 NM_145073.2 NM_178206.2 U62670.1	**Forward primer:** ACCAGAAGTCGACCGAGCTG **Reverse primer:** ATGGCGCACAGGTTGGTGTC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
TOPO1	RT-qPCR		Primer mix (Primerdesign, 26690)	60	95°C, 10 s 60°C, 5 s 72°C, 10 s	45
GAPDH	RT-qPCR	NM_008084.2	**Forward primer:** TGGAGTCTACTGGTGTCTTC **Reverse primer:** GCTGACAATCTTGAGTGAGT	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
NONO	RT-qPCR	NM_023144.2	**Forward primer:(Arsenijevic *et al.*, 2012)** TGCTCCTGTGCCACCTGGTACTC **Reverse primer:** CCGGAGCTGGACGGTTGAATGC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45

Table 3: Oligonucleotide sequences and thermal cycling parameters used for RT-qPCR in samples from *homo sapiens*. Sequences are in the 5'-3' direction as indicated below. Times are given in the order denaturing, annealing and extension.

Gene product	Purpose	Transcript Genbank accession number	Primer sequence (5'---> 3')	Anneal temp (°C)	Cycling parameters	No. of cycles
ERα	RTqPCR	NM_000125.3 NM_001122740.1 NM_001122741.1 NM_001122742.1	**Forward primer:** CTAGAAGGTGGACCTGATCATGG **Reverse primer:** GATGGTCAGTGCCTTGTTGGATG	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
ERβ	RTqPCR	NM_001437.2 NM_001040275.1 NM_001214902.1 NM_001271876.1 NM_001214903.1	**Forward primer:** GATGCGTAATCGCTGCAGACA **Reverse primer:** TGGTGTGAAGCAAGATCGCTAG	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Sirt1	RTqPCR	NM_012238.4	**Forward primer:** GGCAAAGGAGCAGATTAGTAGG **Reverse primer:** CATCAGGCTCATCTTCTAAGCC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3a	RTqPCR	NM_003529.2	**Forward primer:** GCTCGGAAGTCTACTGGTG **Reverse primer:** GGACTTCTGATAACGGCGG	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3b	RTqPCR	NM_003537.3	**Forward primer:** ACCGATCTTCGCTTCCAGAGCTC **Reverse primer:** GTCACTCGCTTAGCATGGATGGC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45

Histone H3c	RTqPCR	NM_003531.2	**Forward primer:** GTCGCTACCAGAAGTCCAC **Reverse primer:** GTGTCTTCGAAGAGTCCCAC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3d	RTqPCR	NM_003530.4	**Forward primer:** ATCCGCAACGACGAGGAG **Reverse primer:** GTGGACTTGCGAGCAGTC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3e	RTqPCR	NM_003532.2	**Forward primer:** GTACTAAGCAGACGGCTCG **Reverse primer:** CTTCTGGTAGCGACGGATC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3f	RTqPCR	NM_021018.2	**Forward primer:** CAGCTCGTAAGTCCACTGG **Reverse primer:** GCGAATCAGTAGCTCAGTCG	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3g	RTqPCR	NM_003534.2	**Forward primer:** CAAGCAGACTGCACGCAAG **Reverse primer:** CTGATAGCGGCGAATCTCG	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3h	RTqPCR	NM_003536.2	**Forward primer:** CTCGGTGGACTTCTGGTAG **Reverse primer:** CGTACGAAGCAGACTGCTC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3i	RTqPCR	NM_003533.2	**Forward primer:** CTACCAGAAGTCGACCGAG **Reverse primer:** CGCACAGGTTGGTATCCTC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45

Histone H3j	RTqPCR	NM_003535.2	**Forward primer:** GACAGCTCGCAAGTCTACC **Reverse primer:** GAGCAGCTCAGTCGACTTC	55	95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3.2	RTqPCR	NM_021059.2	**Forward primer:** CTAAGCAGACTGCTCGCAAGTC **Reverse primer:** GATCAGCAGCTCCGTGGACTT		95°C, 10 s 55°C, 5 s 72°C, 10 s	45
Histone H3.3	RTqPCR	NM_002107.4	**Forward primer:** GCTTCCAGAGCGCAGCTATC **Reverse primer:** CTTAAGCACGTTCTCCACGTATG		95°C, 10 s 55°C, 5 s 72°C, 10 s	45
TOPO1	RTqPCR		Primer mix (Primerdesign, 26690)		95°C, 10 s 55°C, 5 s 72°C, 10 s	45
GAPDH	RTqPCR		**Forward primer:** TGAAGGTCGGAGTCAACGGATTTG **Reverse primer:** CATGTAAACCATGTAGTTGAGGTC		95°C, 5 s 55°C, 5 s 72°C, 10 s	45

Table 4: Oligonucleotide sequence and thermal cycling parameters used for amplification of human ERβ and histone H3.1 (variant H3b) promoter region by PCR. Sequences are in the 5'-3' direction and restriction sites are highlighted in red as indicated below.

Gene/ Product	Purpose	Transcript Genbank accession number	Primer sequence (5'---> 3')	Anneal temp (°C)	Cycling parameters	No. of cycles
ERβ promoter region	PCR amplification	NC_018925.2	**Forward primer:** **CTCAAGCTT**GTGGTGTATGCCTGTAATC **Primer Reverse:** GAGGAATTCGGAAGGTATGTATATGGAGC	55	See section 2.7.2	30
Histone H3 promoter region (H3b)	PCR amplification	M26150	**Forward primer: (2)(Alatawi, 2012)** GTGGGAGAAGTGCCATGCAGCAC **Reverse primer:** CTTGCCTGCAGAGACGTCTGTG	57	See section 2.7.2	40

Table 5: oligonucleotide sequences and thermal cycling parameters used for PCR and pyrosequencing in samples from *mus musculus*. Sequences are in the 5'-3' direction as indicated below. Times are given in the order denaturing, annealing and extension.

Gene	Sequence ID	Primer sequence (5'---> 3')	Anneal temp (°C)	Cycling parameters	No. of cycles
Acaca	NT_096135.6	**Forward primer:** Bio-TGGAATTAATGGAAATGGGATAGG **Reverse primer:** AACTCAACACAACCCTTCATCCT	50	95°C, 30 s 50°C, 30 s 72°C, 90 s 72°C, 120 s	50
Fasn	NT_096135.6	**Forward primer:** Bio-GGATAGGAGGGAAGGGAATAATT **Reverse primer:** AACTCCCACCCTCTATAACCAC	50	95°C, 30 s 50°C, 30 s 72°C, 90 s 72°C, 120 s	50
Sequenced region					
Acaca **Pyrosequencing primer:** TTTTTTTGGTGGTATATATG **Sequenced part:** TTTGC/TGTTAGGGAGTAGTGTTTC/TGTATAGGTGTTTGTC/TGTTGGGTAGGC/TGTTAGTTGTTAAATAC/TGGTTGTTTTTAGTTTGTGTAGTTTTTAGAAA					
Fasn **Pyrosequencing primer:** GGTATTTAGTTAGTTAGATG **Sequenced part:** GTC/TGC/TGTTTGGATATTGAGC/TGGATTTC/TGGAGGTC/TGTTATAC/TGC/TGTTC/TGTTAGTGTTTTTTATTTTGTTTATTGTTT					

Chapter 3

3. The induction of a beige adipocyte phenotype by genistein

3.1. Introduction

Published observations indicate that genistein can affect cell differentiation into adipocytes and also lipid metabolism. For example, a relatively low dose (up to 10 μM) of genistein was found to prevent lipogenesis while at a higher dose (>10 μM) genistein induced lipogenesis in primary mouse bone marrow cells (Dang *et al.*, 2003) and primary human synovial fibroblasts (Relic *et al.*, 2009). In contrast to these in vitro studies, observations made in mice were that at a low dose (50 mg/Kg per day) genistein induced lipogenesis, while at a higher dose (200 mg/Kg per day) genistein inhibited lipogenesis (Penza *et al.*, 2006). Overall, however, it appears that genistein has the potential to affect adipose tissue physiology in a beneficial manner either by inhibiting the production of new adipocytes and/or through effects on adipocyte fat metabolism. In support of this view both in vivo and in vitro studies showed that genistein inhibited the expression of key transcription factors and their downstream genes involved in the process of lipid biogenesis, including peroxisome proliferator-activated receptor-γ (*PPARγ*), sterol regulatory element-binding protein-1 (*SREBP-1*) and fatty acid synthase (*FASN*) (Zhang *et al.*, 2009; Kim *et al.*, 2010) but increased the expression of genes involved in fat catabolism, including *PPARα* and its downstream genes including carnitine palmitoyltransferase 1 (*CPT1*) (Kim *et al.*, 2004).

Genistein appears to have pleiotropic cellular actions mediated through different molecular targets. It is thus difficult to attribute physiological effects to specific actions at the molecular level. However, there is good evidence that genistein can act as an estrogen receptor (ER) ligand. At lower concentrations (up to 3.7 μM) genistein appears to have estrogen-like effects (Chen and Donovan, 2004; Magee and Rowland, 2004) while the action of genistein at higher concentrations seems to be via other cellular pathways, including inhibition of tyrosine kinase and topoisomerase activities (Schmidt *et al.*, 2008). Genistein has also been shown to have epigenetic actions including effects on DNA methylation and histone modification (Zhang *et al.*, 2013a; Xie *et al.*, 2014). In summary, many molecular actions of genistein have been demonstrated and many of these actions are possible actions that may underlie some of the physiological manifestations of

genistein consumption. However, further research in this area is merited to attribute unequivocally specific beneficial outcomes to particular molecular actions.

White adipocytes synthesize and store fat in the form of triglycerides and release it as a source of energy as required (Lafontan, 2005) depending on nutritional status. When energy availability is high white adipocytes store energy in the form of triglycerides and release it in the form of free fatty acids during periods of energy deprivation (Sorisky, 1999). White adipocytes are highly active and responsive (Kim and Moustaid-Moussa, 2000). They actively communicate with other cells by receiving, synthesizing and sending biological signals in the form of biologically active peptides and proteins, which are known as adipokines. Through adipokines, adipocytes play important roles in integrating and regulating the overall physiological and metabolic activity of adipose tissue and other organs (Frühbeck et al., 2001; Ali et al., 2013).

In contrast to white adipose tissue, the presence of brown adipose tissue reduces fat storage. Brown adipose tissue functions metabolically to dissipate energy in the form of heat (Lowell and Spiegelman, 2000). Brown adipocytes are characterised by having small multi-locular fat droplets (Cinti *et al.*, 1997; Ravussin and Galgani, 2011; Lee *et al.*, 2012c) and the cytoplasm of brown adipocytes is densely packed with mitochondria. The mitochondria of brown adipocytes can be distinguished from those of white adipocytes by the presence of uncoupling protein-1 (UCP1), which is responsible for dispersing stored energy as heat by uncoupling ATP synthesis from electron transport (Matthias et al., 2000; Cannon and Nedergaard, 2004; Frontini et al., 2007). Absence of or defects in UCP1 activity in brown adipose tissue have been seen to be associated with obesity, metabolic disorders (Nedergaard *et al.*, 2007; Cannon and Nedergaard, 2009) and defective adaptive thermogenesis (Golozoubova et al., 2001; Meyer et al., 2010) in humans and mice. Development of brown/beige adipose tissue within the white adipose tissue depots of the body may be considered one of the important approaches to reduce obesity and its related disorders including diabetes, cardiovascular disease and cancer.

Brown adipocytes are characterised by expressing a distinct set of genes that are involved in the process of differentiation (*SIRT1, C/EBPβ, PPARGC1A*) or confer the specialised function of non-shivering thermogenesis (*UCP1*). Additionally, these genes are a useful research tool since they can be used to distinguish brown adipocytes from other cell types and to distinguish between classical brown and beige adipocytes.

Sirt1 appears to have pivotal roles in the development of beige adipocytes as an increase in the expression of Sirt1 in a transgenic mouse model increased the expression level of brown adipocyte marker genes, including *Ucp1* and *Cebpb*, among white adipose tissue depots while the white adipocyte marker genes, *Rarres2* (chemerin) and *Retn* (resistin), were found to be reduced (Qiang et al., 2012). The activities of Sirt1 and PGC1α are closely linked (Aquilano et al., 2010). An increase in the expression level of Sirt1 has been found to enhance the browning of white adipocytes coincident with PGC1α deacetylation in mouse and 3T3-L1 cells (Fu et al., 2014). In addition, PGC1α is a key stimulating factor for mitochondrial biogenesis, which occurs at a high level during the development of brown adipocytes (Rodgers et al., 2005; Guarente, 2007; Fernandez-Marcos and Auwerx, 2011). PGC1α appears to have a stimulatory effect on *UCP1* expression through recruitment of PPARγ and the thyroid receptor onto the *UCP1* promoter (Puigserver et al., 1998; Tiraby et al., 2003). In the same way, PGC1α acts as a coactivator of PPARα to increase UCP1 expression (Barberá et al., 2001) and it also associates with the retinoic acid receptor to augment UCP1 expression (Liang and Ward, 2006).

Recently, C/EBPβ was recognised as a conspicuous player in the development of classical brown and beige adipocytes and has been used as a characteristic marker of brown adipocytes (Qiang et al., 2012). Its expression appears to be critical for differentiation of brown adipose tissue in mice (Tanaka et al., 1997). In addition, overexpression of C/EBPβ in human skin fibroblasts, mouse skin fibroblasts and 3T3-L1 cells was shown to be sufficient to convert these cells into brown adipocytes (Karamanlidis et al., 2007; Kajimura et al., 2009). Consistently, the C/EBPβ deficient mouse embryo was reported to have depleted brown adipose tissue and a reduction in the expression level of UCP1 (Kajimura et al., 2009). In addition, defective thermogenesis was observed in the C/EBPβ knockout mouse (Carmona et al., 2005).

UCP1 appears to be the protein primarily responsible for the induction of non-shivering thermogenesis and it is expressed selectively by brown adipocytes including both classical brown and beige adipocytes (Golozoubova et al., 2001; Golozoubova et al., 2006). However, CD-137 has been discovered recently to be selectively expressed in beige rather than classical brown adipocytes (Wu et al., 2012; Fu et al., 2014) and its expression can be used to differentiate beige adipocytes from classical brown adipocytes. CD-137 has

been used to classify some brown adipocyte depots, including human epicardial and visceral paracardial fat depots, as beige rather than classical brown adipose tissue (Sacks et al., 2013).

3.2. Objectives

The initial objective of this research was to address the hypothesis that genistein may affect the expression of genes linked to the process of lipogenesis, specifically *Fasn* and *Acaca,* on the basis of previous observations made in the laboratory by Goiuri Alberdi Aresti who found a reduction in the mRNA levels of lipogenic enzymes comprising FAS and ACC in 3T3-L1 cells after treatment with resveratrol. I proposed that other polyphenols that, like resveratrol, resemble estrogen-in particular genistein-may enhance a similar change in gene expression. The approach was to treat the 3T3-L1 cell line, which can be induced to differentiate into adipocytes, with genistein then extract mRNA to measure transcript levels by RT-qPCR (primer sequences are stated in chapter 2, table 1). The work led to the serendipitous discovery that genistein appears to promote the appearance of brown/beige rather than white adipocytes, which may be through a process of either trans-differentiation or by promoting differentiation from pre-adipocytes with a brown adipocyte-like phenotype rather than cells with a white adipocyte phenotype. Thus the major objective was to investigate if genistein could regulate the expression of genes that characterise both white (*Acaca, Fasn, Fabp4, Lipe, Rarres2, Retn*) and brown (*Ucp1, Tnfrsf9*) adipocytes, and/or affect the expression of genes that have been recognised as mediators of white to brown adipocyte interconversion (*Sirt1, Cebpb, Ppargc1a*).

To probe further the effect of genistein I observed and to gain an indication of functional consequence, mitochondrial function was also measured.

3.3. Results

3.3. 1. Differentiation of 3T3-L1 cells

3T3-L1 cells were stimulated to differentiate into mature adipocytes using a standard published protocol used by other researchers (Caprio *et al.*, 2007; Arsenijevic *et al.*, 2012) as discussed in chapter 2, section 2.1.1. Staining of cells with oil red-O stain and measurement of mRNA levels of genes that characterise white adipocytes comprising *Acaca*, *Fasn*, *Fabp4* and *Lipe* by RT-qPCR at day 0 and day 12 of differentiation determined that this procedure led to development of an adipocyte-like phenotype. Fat accumulation within the cells was markedly increased after 12 days of differentiation (Figure 3.1) and expression of *Acaca, Fasn, Fabp4* and *Lipe* mRNA was increased by several fold at day 12 in comparison to day 0 (Figure 3.2).

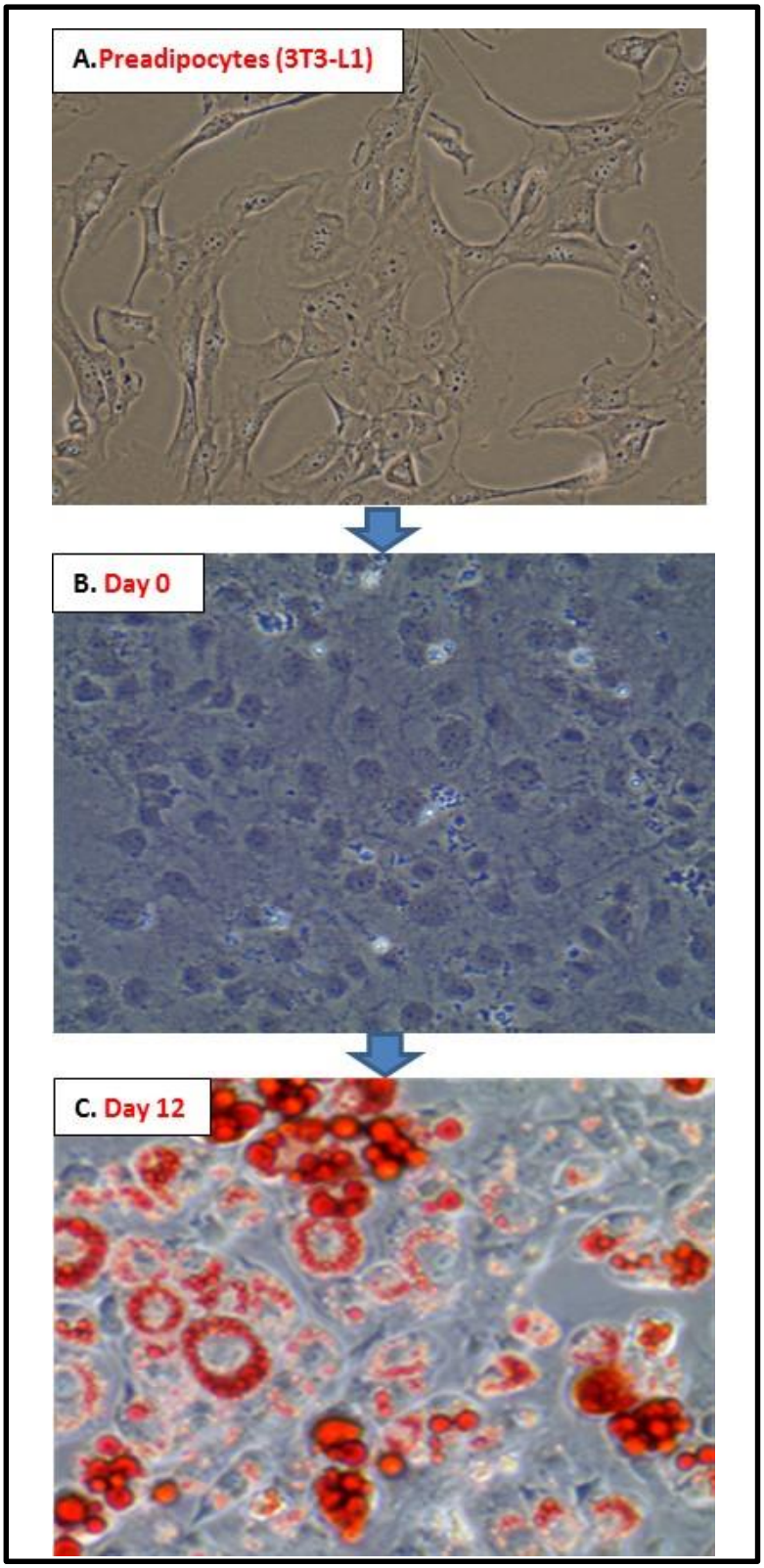


Figure 3. 1: Growth and differentiation of 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate. Cells were fixed in 10% neutral formaldehyde and stained with Oil Red-O stain on day 0 and day 12 to reveal fat droplets. Cells were photographed using a 40X objective lens. Initially 3T3 cells had a fibroblastic-shape (A), became rounded after reaching confluence (B), and on day 12 of differentiation showed a marked accumulation of fat droplets within the cytoplasm, indicating a mature white adipocyte phenotype (C).

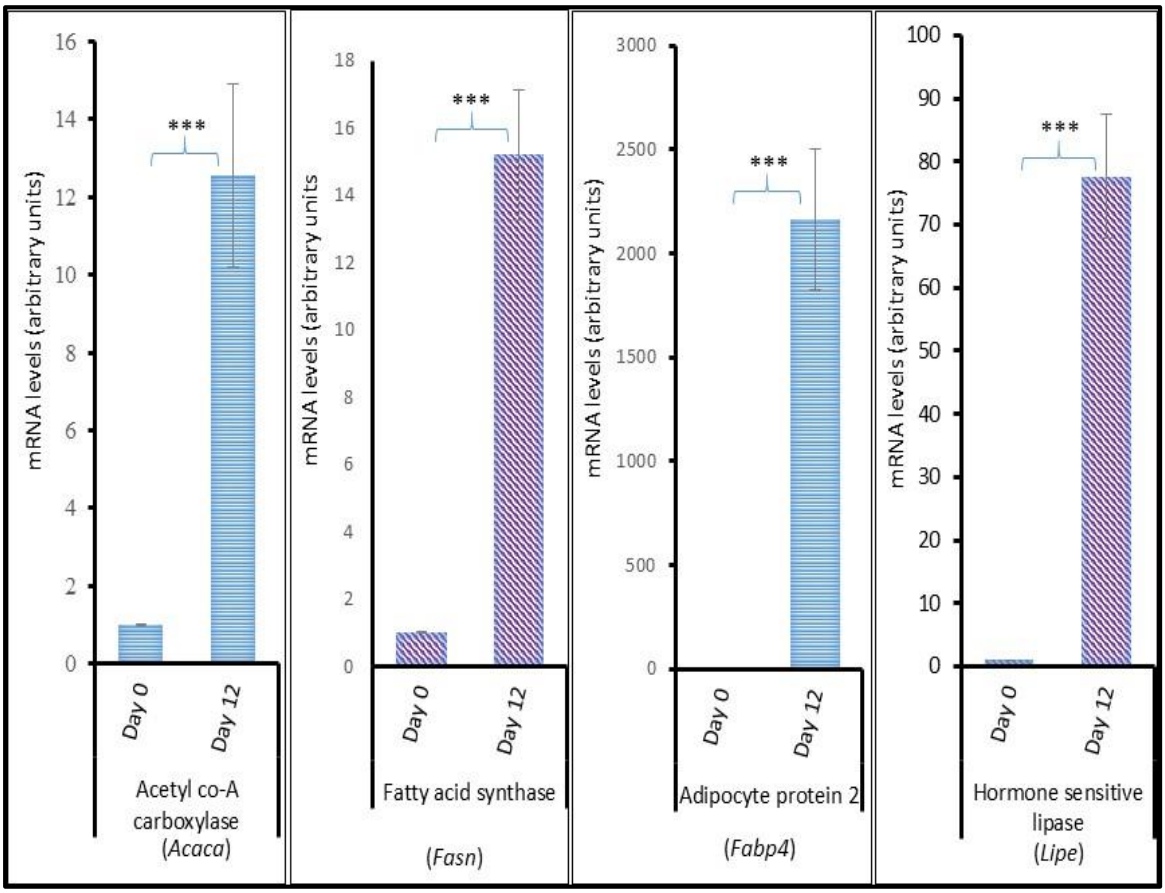


Figure 3. 2: The mRNA levels of gene products that characterise white adipocytes (ACC, FAS, AP2 and HSL) in 3T3-L1 cells on day 0 and day 12 of differentiation. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day-0, cells were induced to differentiate. Total RNA was extracted on day 0 and day 12. mRNA expression levels were measured by RT-qPCR. Data are expressed relative to TOPO1 and NONO and are normalised to the measurement for day 0. Values are means (n=6 "three separate wells for each of two independent experiments"), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. ***P≤ 0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

3.3.2. Effects of genistein in 3T3-L1 cells on gene expression and lipid accumulation

When 3T3-L1 cells were treated with 50 µM genistein for 48 h beginning on day 10 after inducing differentiation, the expression of genes corresponding to enzymes involved in lipogenesis (*Acaca, Fasn*) and genes expressed in maturing white adipocytes (*Fabp4, Lipe, Rarres2 and Retn*) were upregulated as indicated by measurement of mRNA levels by RT-qPCR. In contrast, 100 µM genistein applied in exactly the same way had an opposite effect and led to down-regulation of the expression of all of these genes with the exception of *Acaca* (Figure 3.3). Parallel effects of genistein on Sirt1 mRNA were very small, but there was a significant reduction with 50 µM genistein and a significant increase with 100 µM genistein (Figure 3.4). C/EBPβ mRNA was not affected by 50 µM genistein but was increased by 100 µM genistein. PGC1α mRNA was reduced by 50 µM genistein (Figure 3.4). UCP1 mRNA was not affected by treatment with either concentration of genistein (Figure 3.4).

To obtain a more detailed and dynamic profile of the changes in gene expression induced by genistein, cells were induced to differentiate in the presence and absence of genistein (10 µM, 50 µM and 100 µM) and the expression of the same genes was measured at day 2, day 3, day 4, day 5, day 8 and day 12 by RT-qPCR. I found that effects of genistein on these mRNAs was dose and time dependent such that mRNAs corresponding to genes involved in lipogenesis (*Fasn, Acaca*) and genes expressed in maturing adipocytes (*Fabp4, Lipe, Rarres2 and Retn*) reached maximum levels at lower concentrations of genistein over a longer treatment period or at higher concentrations of genistein more quickly, and then declined.

Observation of the cells after staining with Oil red-O stain revealed that higher doses of genistein or treatment over a long period of time led to an accumulation of small multilocular fat droplets within the cytoplasm of the cells, which contrasted with the typical large fat droplets observed in the control differentiated cells (Figure 3.5). The accumulation of small multilocular fat droplets is considered one of the characteristic features of brown adipocytes.

Figures (3.6 to 3.16) and appendix (2) show the response to each concentration of genistein applied as mRNA measured at each time point. Genistein (10 µM) treatment caused the expression of genes involved in white adipocyte maturation (*Fabp4, Lipe,*

Rarres2, Rent) to increase at day 4. Expression of genes involved in lipogenesis (*Acaca, Fasn*) then increased, being evident at day 8, at which point expression of the genes involved in white adipocyte maturation (*Fabp4, Lipe, Rarres2, Rent*) had further increased. However, these differences were not observed at day-12, with the exception of the response of *Lipe* and *Rent*. The expression of genes involved in mediating white to brown adipocyte interconversion (*Sirt1, Cebpb*) was not affected, while there was a reduction in the expression of *Ppargc1α* on day 8 and day 12.

Higher doses of genistein, specifically 50 µM genistein, were found to increase expression of genes involved in lipogenesis (*Acaca, Fasn*) and white adipocyte maturation (*Fabp4, Lipe, Rarres2, Rent*) at earlier time points of the treatment (day 2, day 3 and day 5) these increase were followed by a dramatic reduction in expression (day 8 and day 12). Sirt-1 mRNA was significantly decreased (day 8 and day 12) after an initial increase (day 2). There was a persistent increase in C/EBPβ and PGC1α mRNA, particularly after day 5 of the treatment, while the mRNA of markers of beige adipocytes, *Ucp1* and *Tnfrsf9,* fluctuated.

The expression of genes involved in lipogenesis and white adipocyte maturation, specifically *Fasn, Fabp4, Lipe, Rarres2* and *Rent,* was markedly decreased by 100 µM genistein at the earliest time point measured (day 2). In addition, there was a persistent increase in the mRNA of both *Sirt1* and *Cebpb*. After a dramatic increase in the expression of *Ppargc1α* (between day 2 and day 4), there was a gradual decrease in its expression, especially after day 5 of the treatment. The mRNAs for uncoupling protein-1 (UCP1) and CD-137 were significantly increased following treatment with 100 µM genistein, particularly at the later time points.

Figure 3.24 is a heat map that provides a visual overview of these response patterns. The overall relationship between these responses is consistent with genistein promoting the conversion of white adipocytes to beige adipocytes. As genistein concentration increased the observed reduction in the expression of genes involved in lipogenesis (*Fasn*) and/or characteristic of white adipocytes (*Fabp4, Lipe, Rarres2 and Retn*) occurred more rapidly. The observed increase in the expression of genes that mediate the conversion of white to beige adipocytes (*Sirt1, Cebpb, Ppargc1a*) peaked more rapidly. The expression of *Sirt1* and *Cebpb* appears to be persistent throughout the treatment course while *Ppargc1α* was sharply reduced after a dramatic increase. The

increase in the expression of genes involved in mediating white to brown adipocyte interconversion was followed by a peak in the expression of genes characteristic of beige adipocytes including *Ucp1* and *Tnfrsf9*. At each concentration of genistein applied a peak in the expression of genes that play a role in mediating the conversion of white to beige adipocytes (*Sirt1, Cebpb, Ppargc1a*) was followed by a reduction in the expression of genes characterise white adipocytes (*Fabp4, Lipe, Rarres2 and Retn)*.

A notable exception to this pattern of genes responses consistent with genistein promoting a beige adipocyte gene expression profile was *Acaca*, which initially increased after the application of genistein, but were ultimately (like the other markers of white adipocytes) down-regulated. Acetyl Co-A carboxylase (ACC) catalyses the carboxylation of acetyl Co-A to generate malonyl Co-A, an early step in the process of lipogenesis. The metabolic rationale for this change is not clear, and a search of current literature was not revealed any evidence that ACC has a particular role in adipocyte browning, exploring this question in depth would be an interesting topic for future research and may lead to better understanding distinct metabolic activities of the different types of adipocyte tissue.

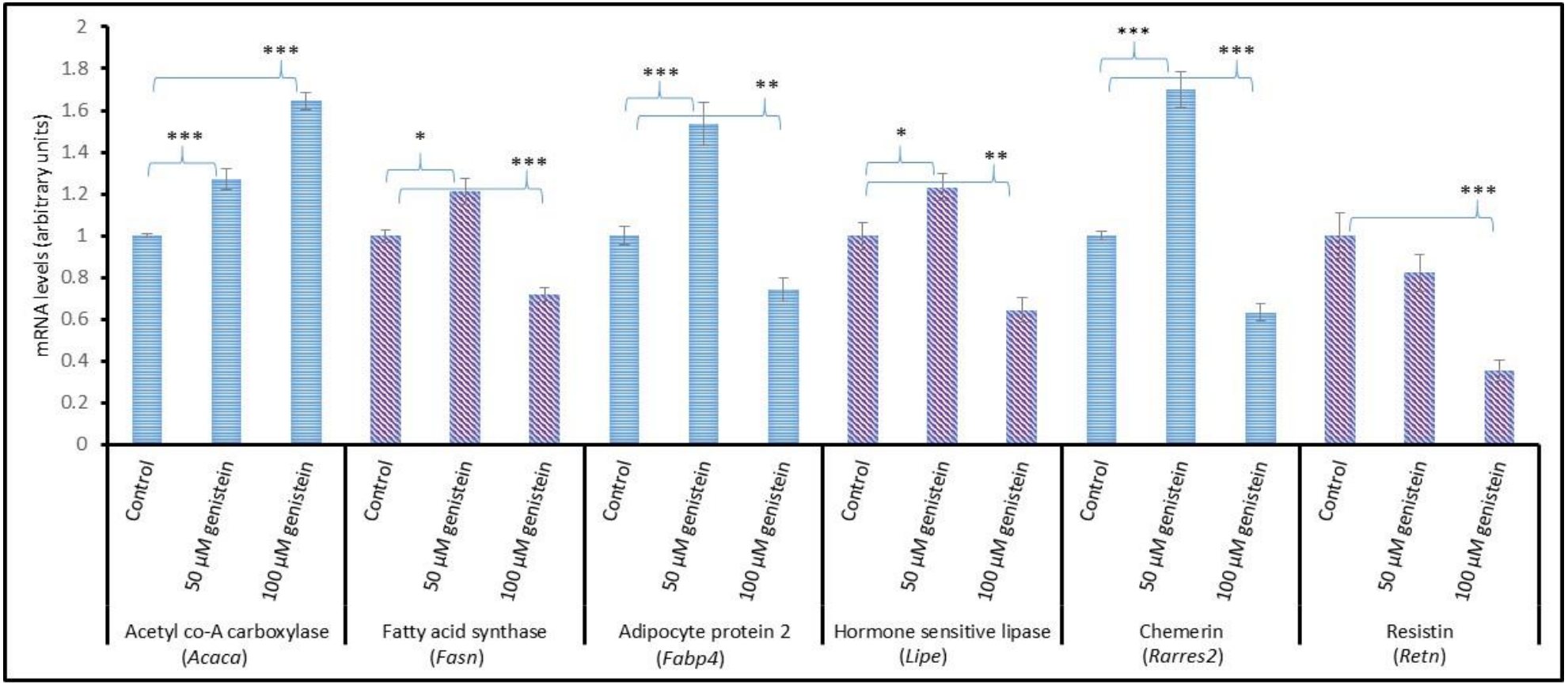


Figure 3. 3: The effect of genistein applied between days 10 and day 12 after inducing differentiation of 3T3-L1 cells on mRNAs corresponding to gene products that are involved in lipogenesis (ACC and FAS) or maturation of white adipocytes (AP2, HSL, chemerin and resistin). Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate. On day 10 cells were treated with genistein (50 µM or 100 µM) for 48 h. Total RNA was extracted on day 12 and mRNA expression levels were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and NONO and normalised to control. Values are means (n=6 "three separate wells for each of two independent experiments"), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

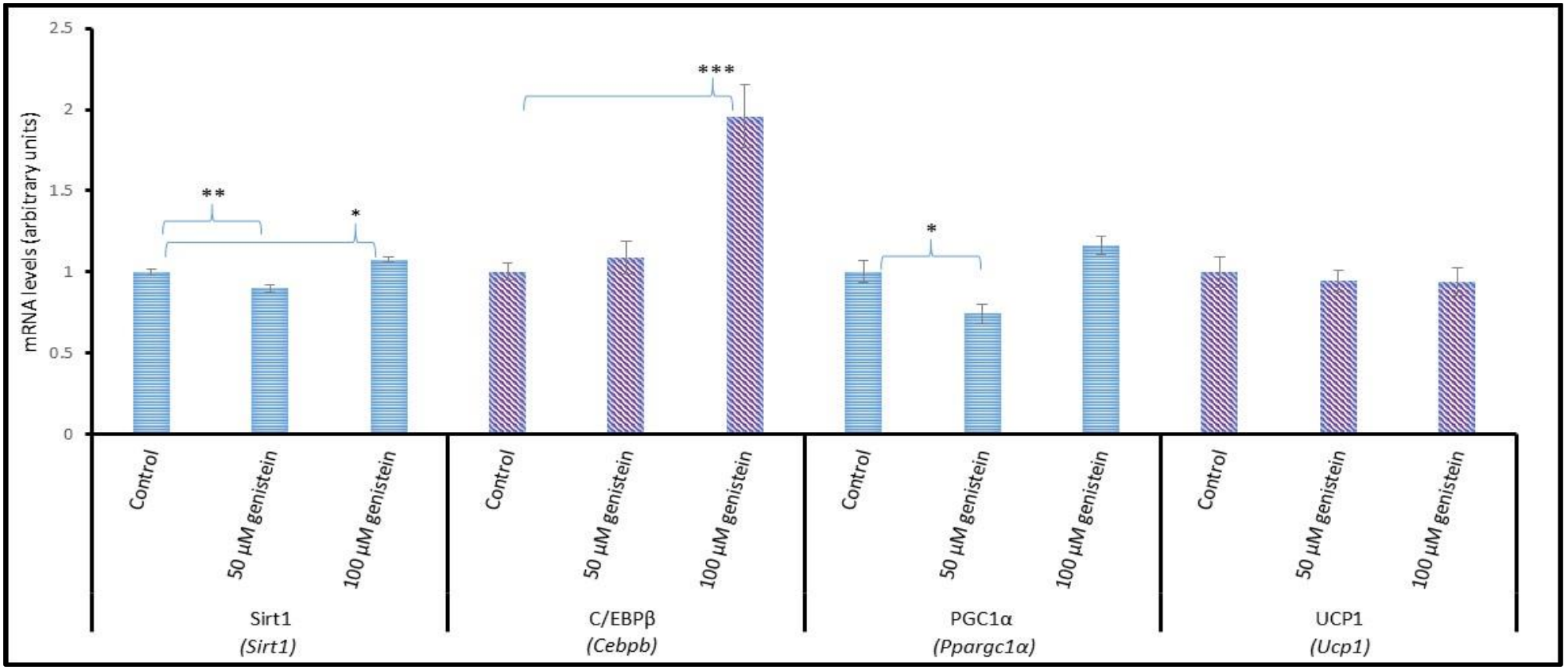


Figure 3. 4: The effect of genistein applied between days 10 and day 12 after inducing differentiation 3T3-L1 cells on mRNAs corresponding to gene products that are involved in interconversion of white to beige adipocytes Sirt1, C/EBPβ and PGC1α) or marks of beige adipocytes (UCP1). Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate. On day 10 cells were treated with genistein (50 µM or 100 µM) for 48 h. Total RNA was extracted on day 12 and mRNA levels were measured by RT-qPCR. Data are expressed relative to the reference genes *Top1* and *Nono* and normalised to control. Values are means, n=6 groups based on 3 technical replicates (wells from the same experiment) for 2 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

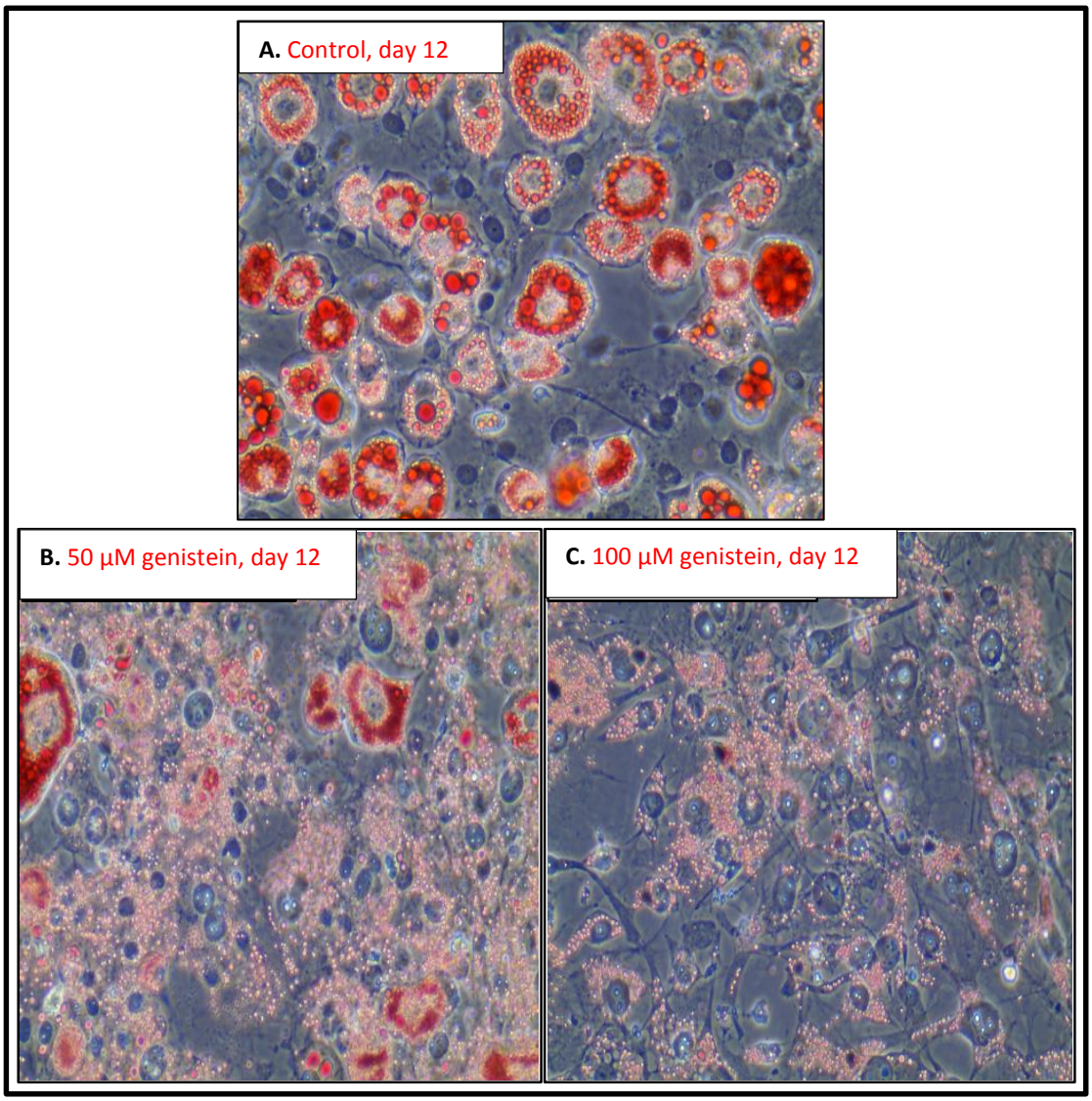


Figure 3. 5: Growth and differentiation of 3T3-L1 cells in the presence and absence of genistein (50 µM or 100 µM). Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate. Cells were fixed in 10% neutral formaldehyde, and stained with Oil Red-O stain on day 0 and day 12 to reveal fat droplets. Cells were photographed using a 40X objective lens. On day 12 of differentiation showed a marked accumulation of fat droplets within the cytoplasm, indicating a mature white adipocyte phenotype (A). In contrast, cells treated with 50 µM or 100 µM genistein had smaller multilocular fat droplets (B, C).

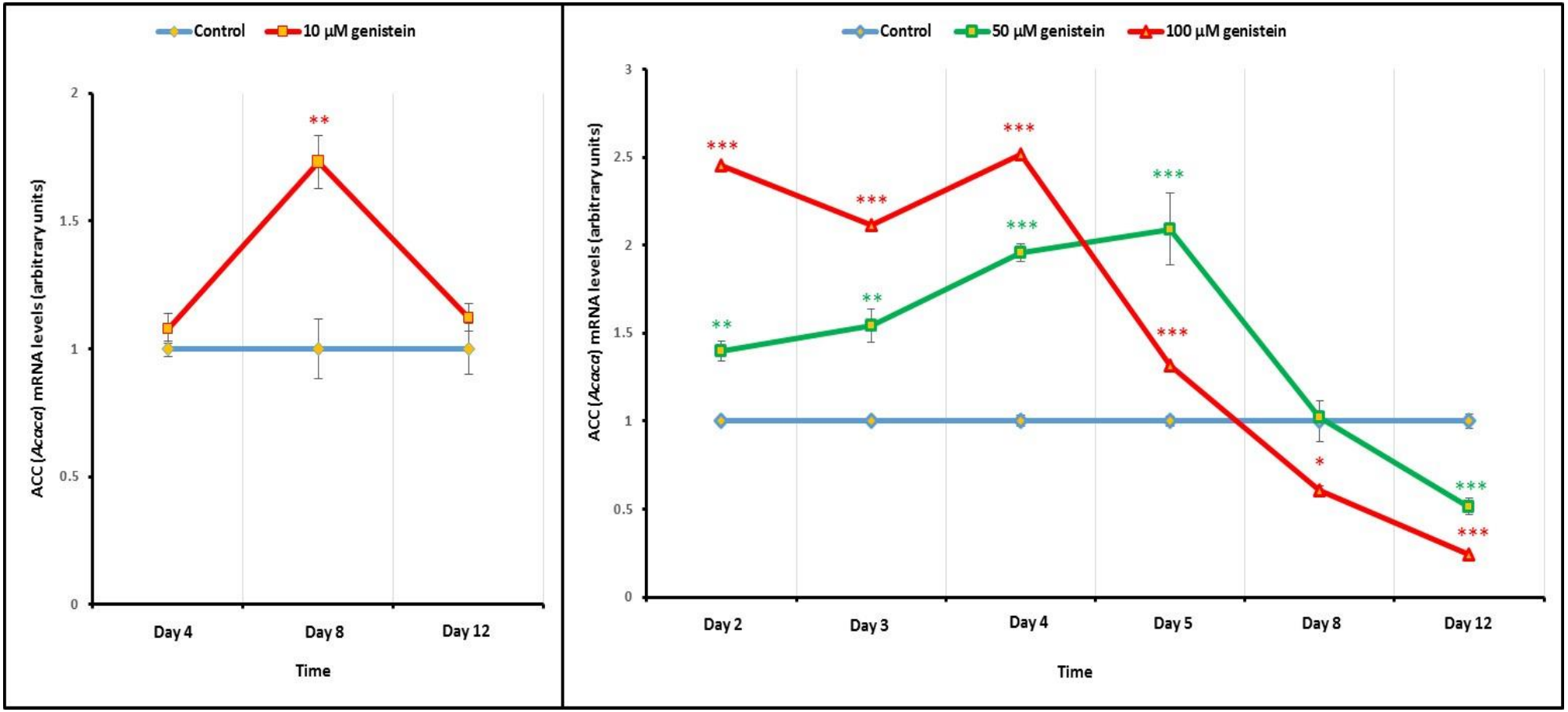


Figure 3. 6: The effect of genistein (10 µM 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to ACC. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted. mRNA was measured by RT-qPCR. Data are expressed relative to the reference genes *Top1* and *Nono* and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤0.05, **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

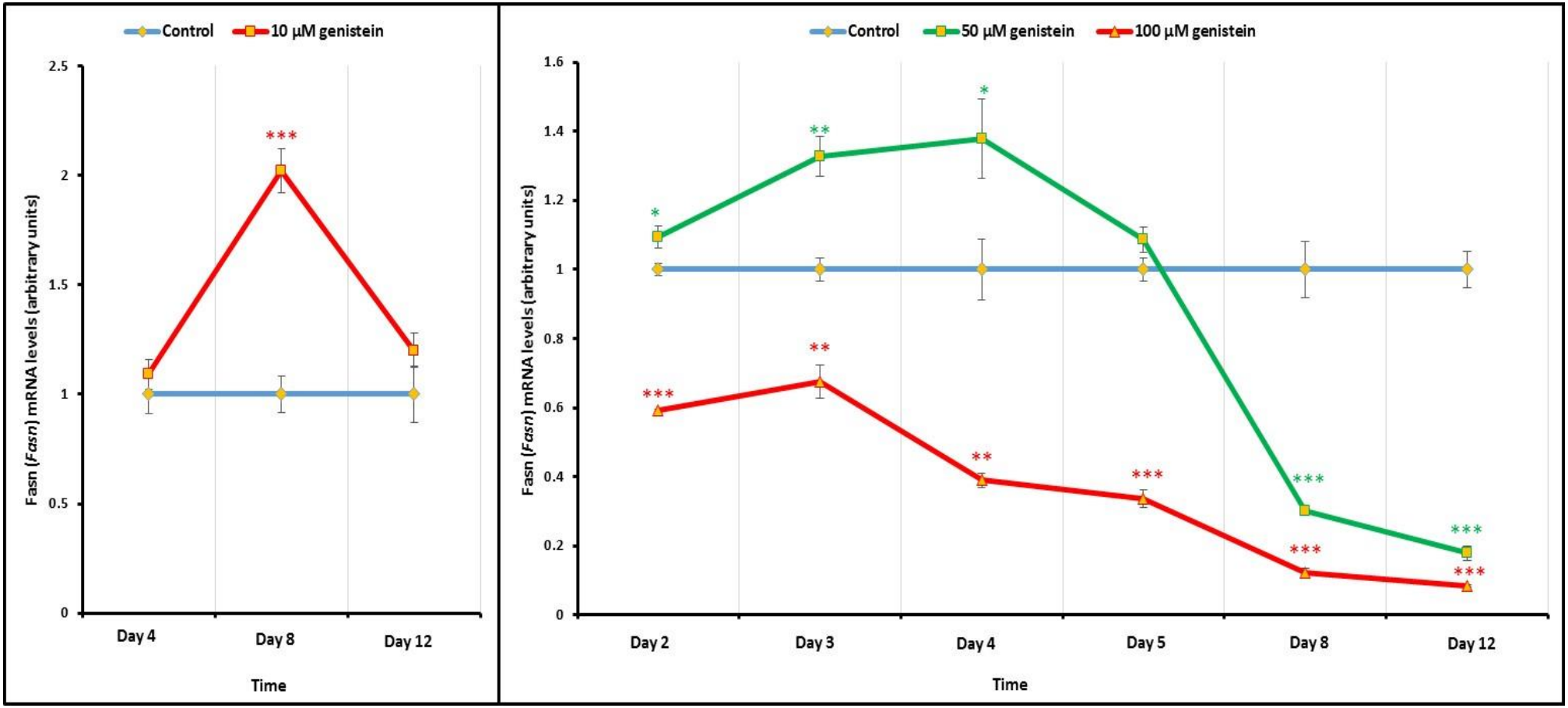


Figure 3. 7: The effect of genistein (10 µM 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to FAS. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted. mRNA expression levels were measured by RT-qPCR. Data are expressed relative to the reference genes *Top1* and *Nono* and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤0.05, **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

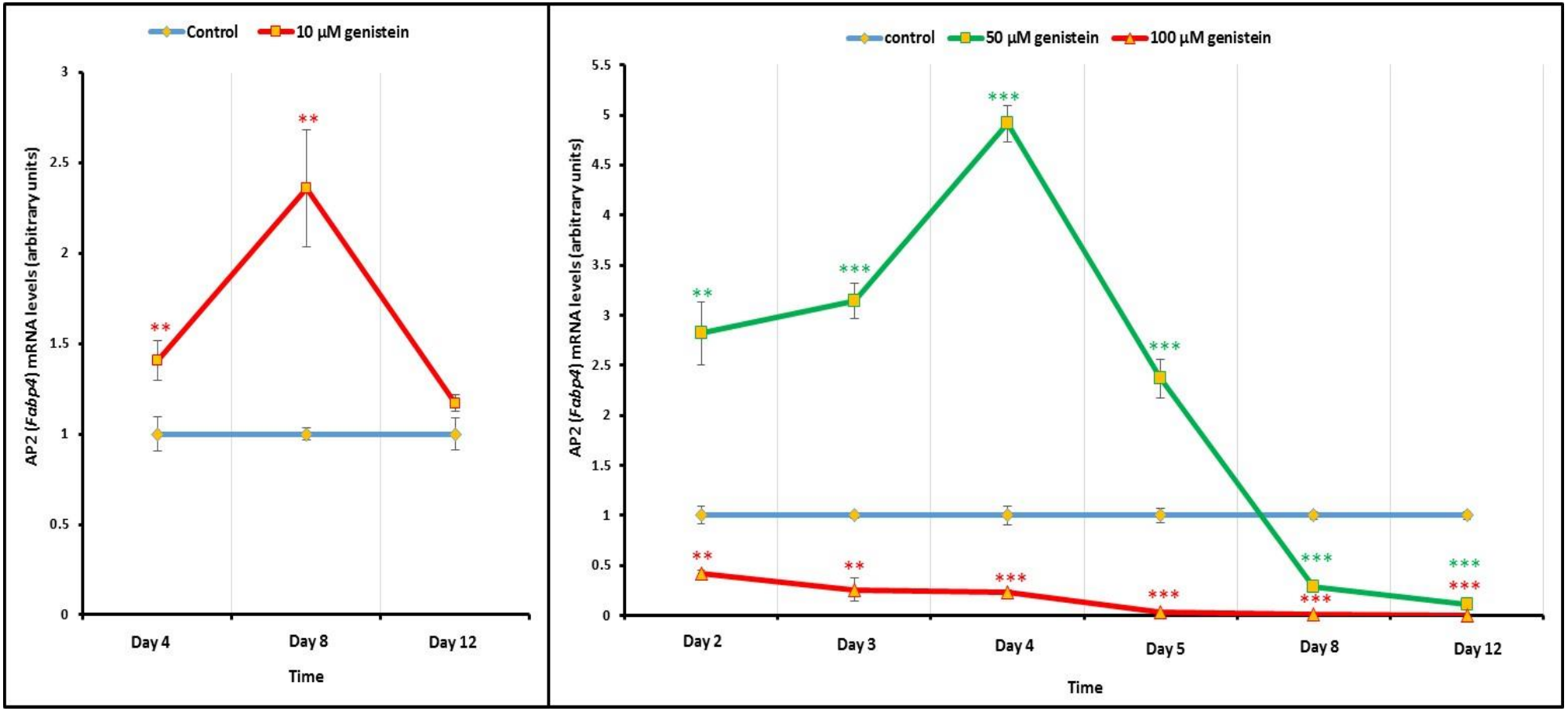


Figure 3. 8: The effect of genistein (10 µM 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to AP2. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted. mRNA levels were measured by RT-qPCR. Data are expressed relative to the reference genes *Top1* and *Nono* and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤0.05, **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

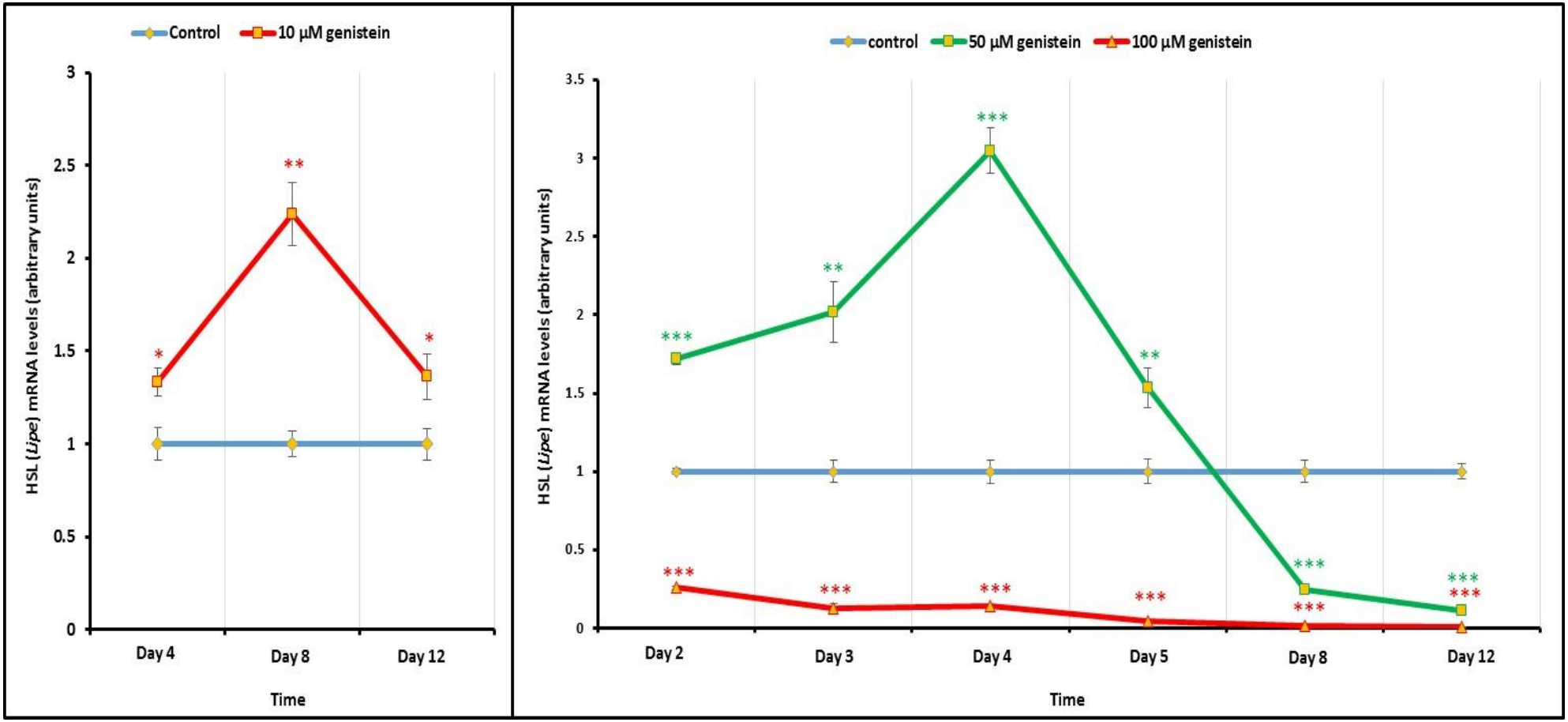


Figure 3. 9: The effect of genistein (10 µM, 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to HSL. Cells were cultured and grown to confluence in 6-well plates, Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted and mRNA levels were measured by RT-qPCR. Data are expressed relative to the reference genes Top1 and Nono and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

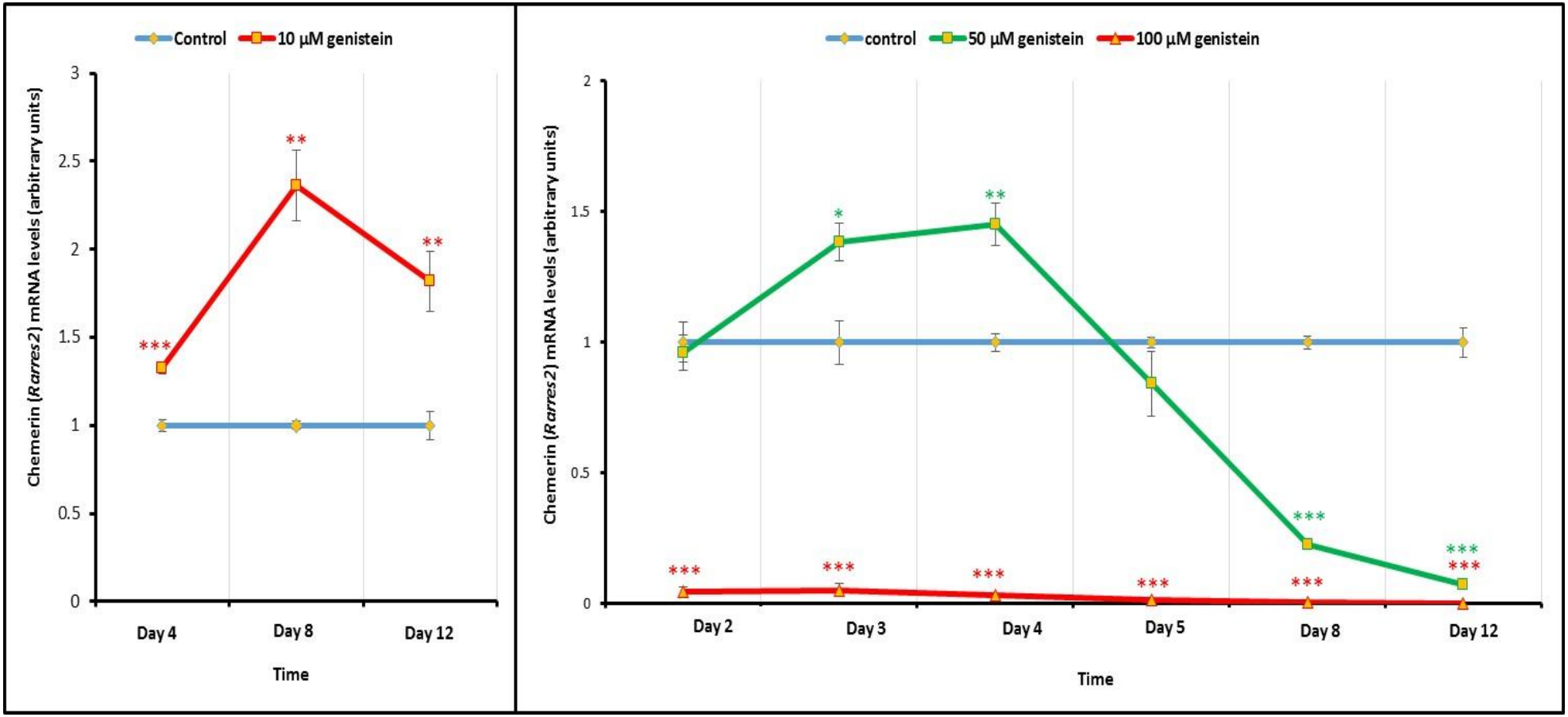


Figure 3. 10: The effect of genistein (10 µM, 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to chemerin. Cells were cultured and grown to confluence in 6-well plates, Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted and mRNA levels were measured by RT-qPCR. Data are expressed relative to the reference genes *Top1* and *Nono* and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

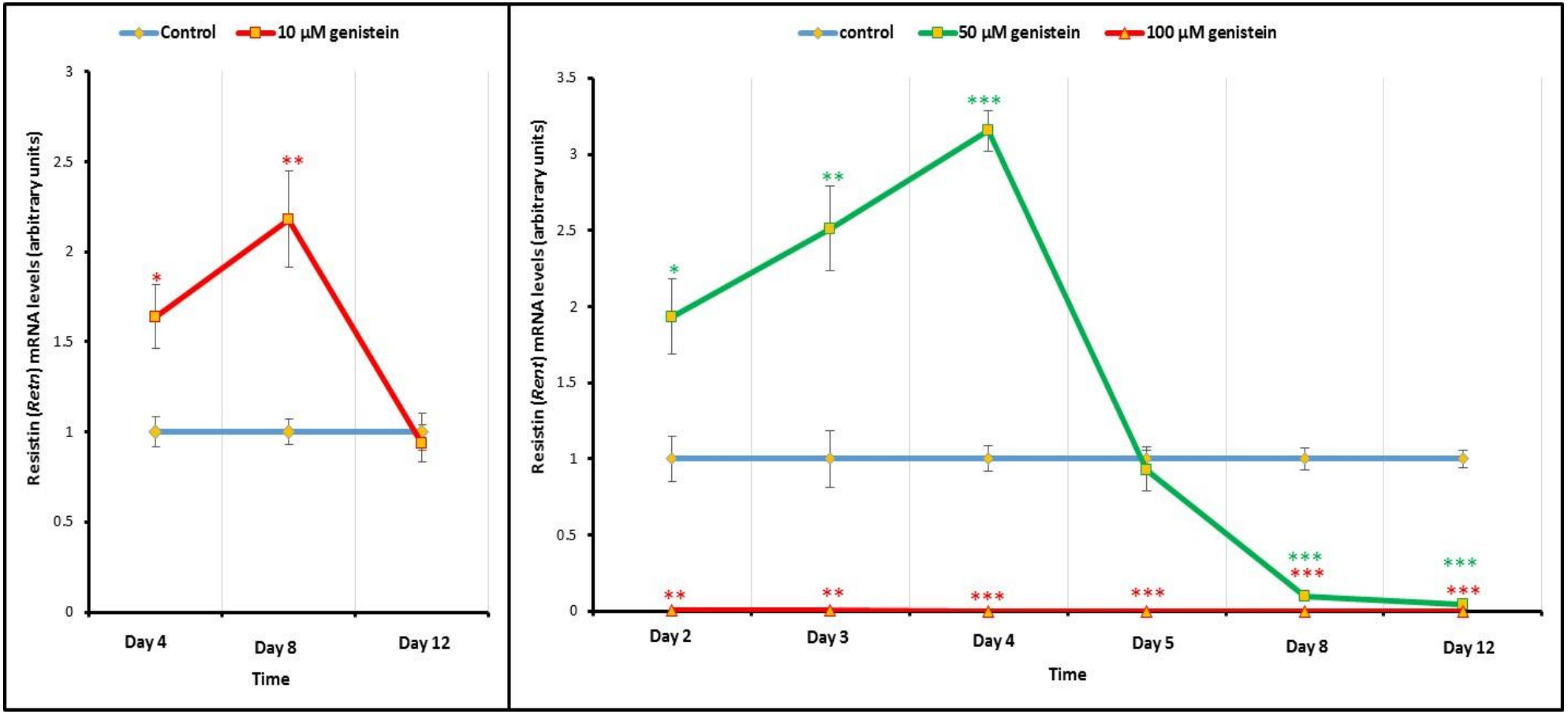


Figure 3. 11: The effect of genistein (10 µM, 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to resistin. Cells were cultured and grown to confluence in 6-well plates, Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted and mRNA levels were measured by RT-qPCR. Data are expressed relative to the reference genes Top1 and Nono and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

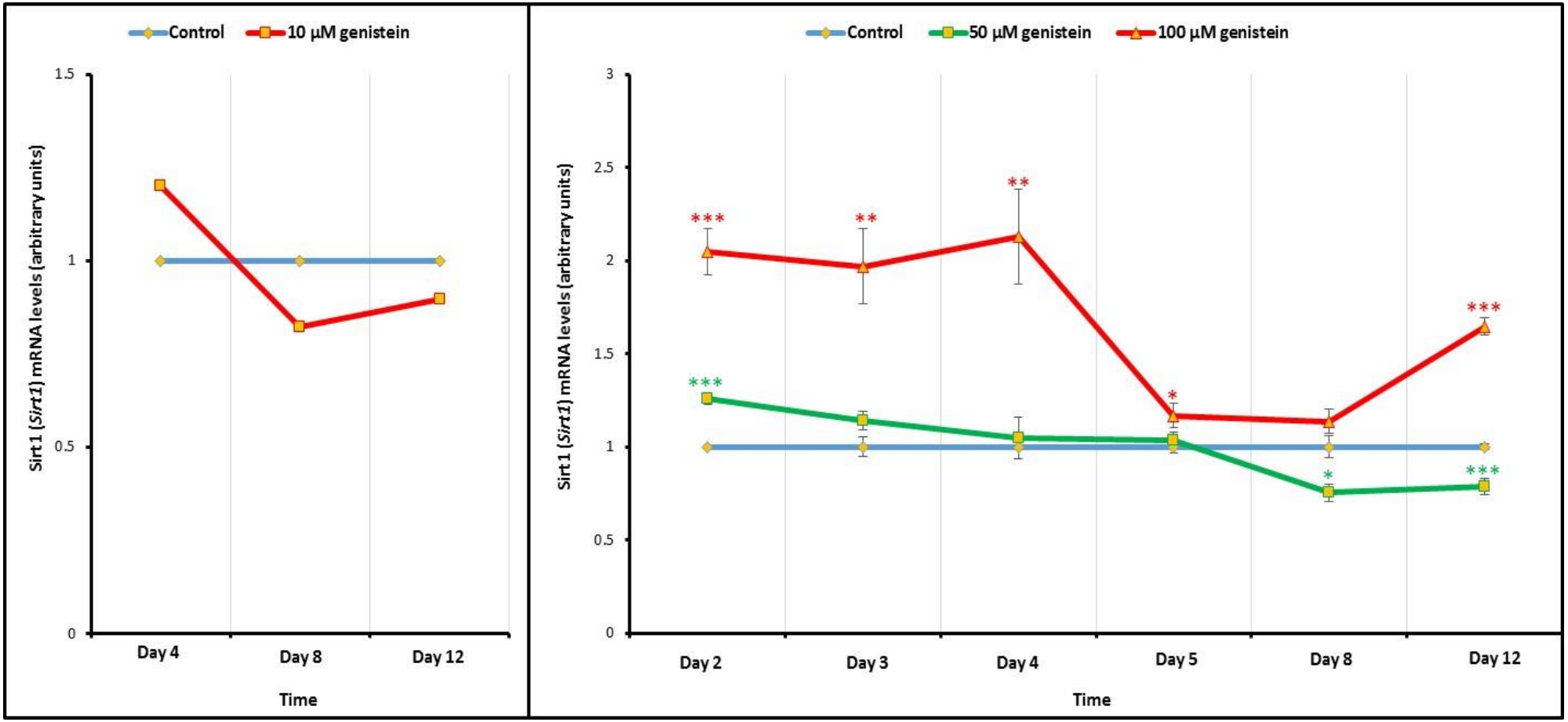


Figure 3. 12: The effect of genistein (10 µM, 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to Sirt1. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted. mRNA was measured by RT-qPCR. Data are expressed relative to the reference genes Top1 and Nono and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤0.05, **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

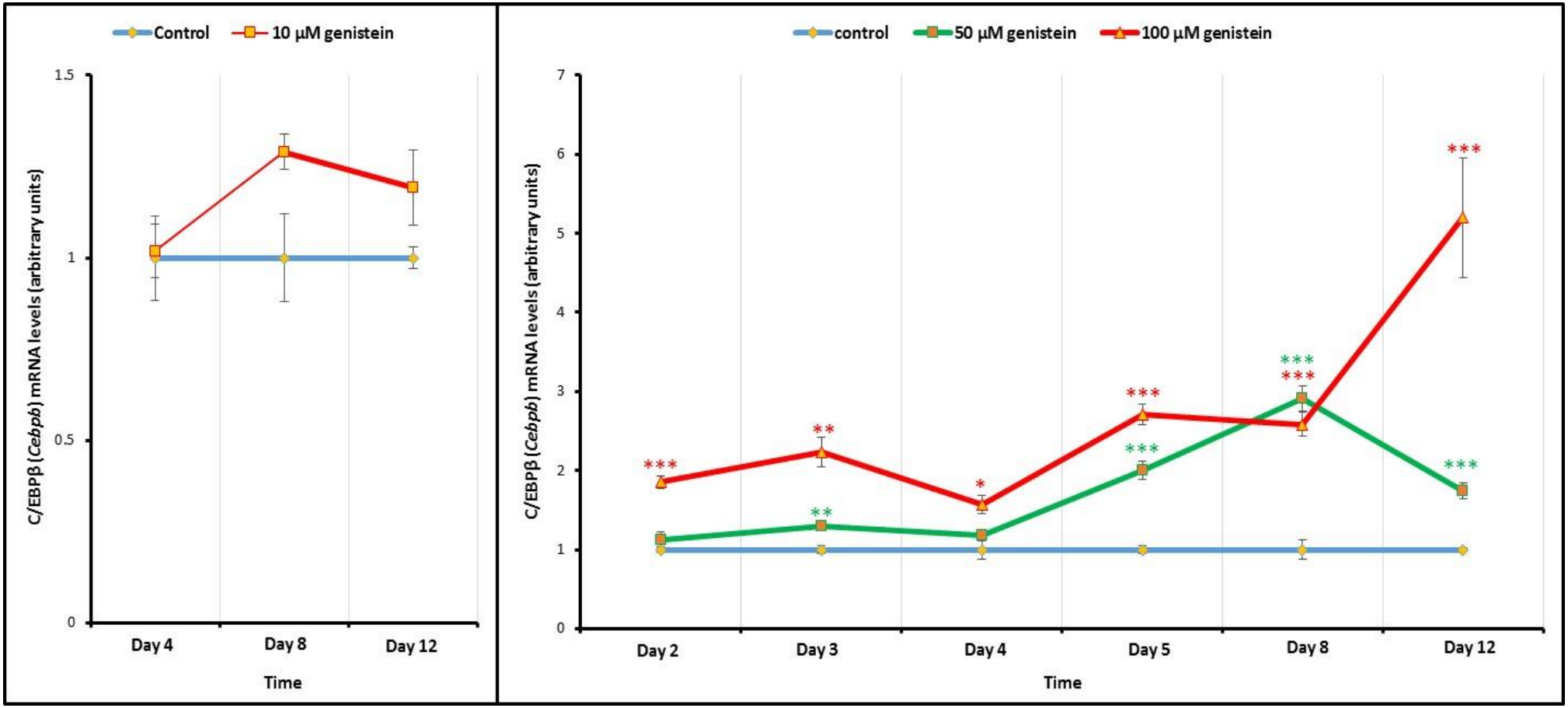


Figure 3. 13: The effect of genistein (10 µM, 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to C/EBPβ. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted. mRNA was measured by RT-qPCR. Data are expressed relative to the reference genes Top1 and Nono and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤0.05, **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

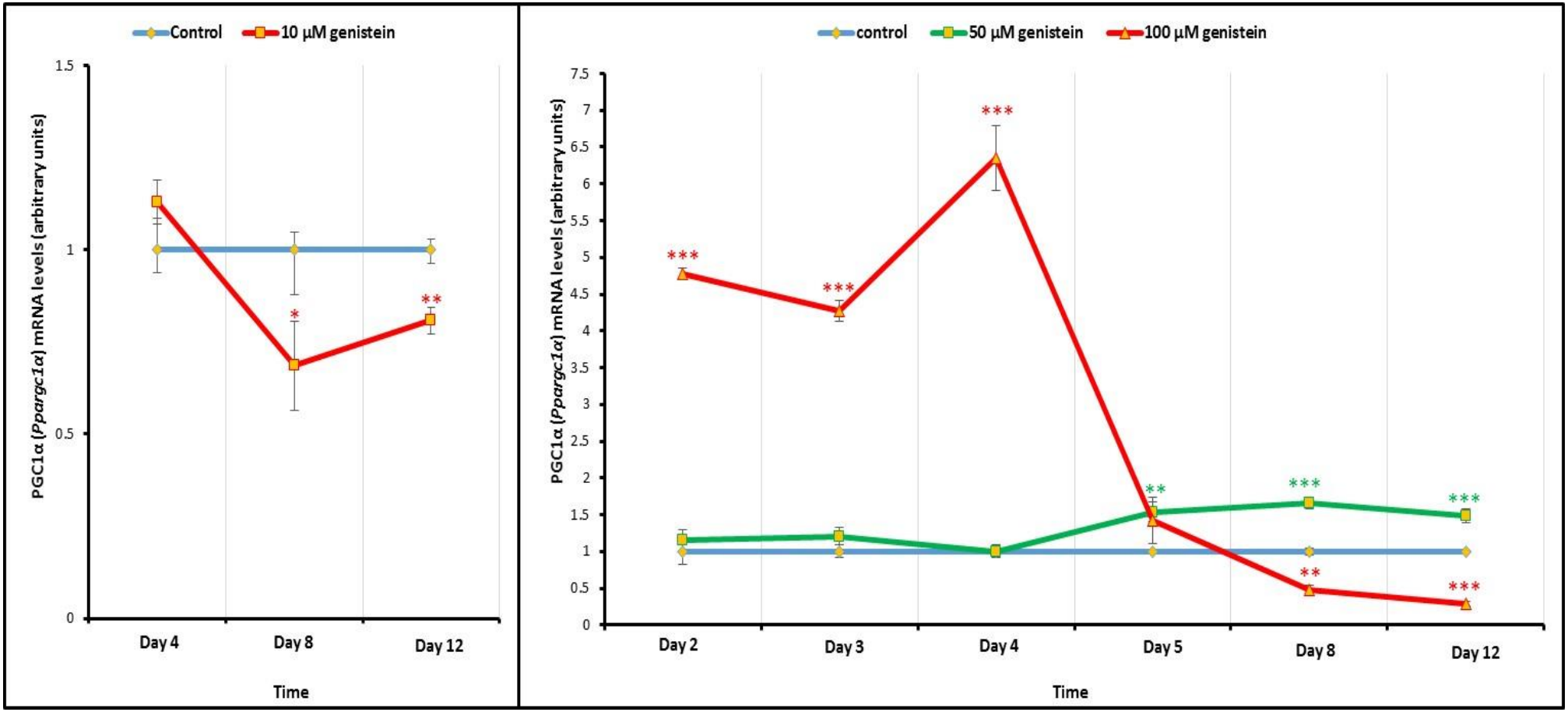


Figure 3. 14: The effect of genistein (10 µM, 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to PGC1α. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted. mRNA was measured by RT-qPCR. Data are expressed relative to the reference genes *Top1* and *Nono* and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤0.05, **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

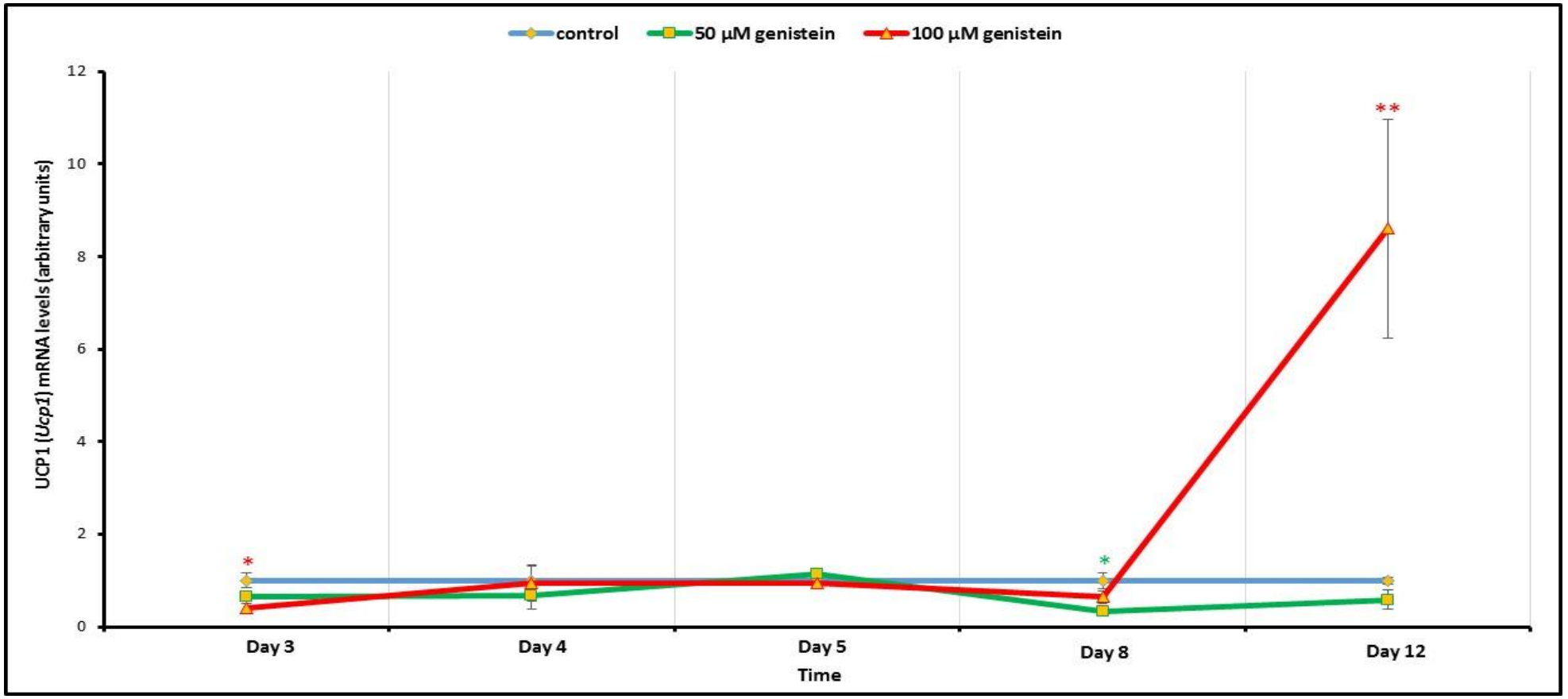


Figure 3. 15: The effect of genistein (10 µM, 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to UCP1. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted. mRNA was measured by RT-qPCR. Data are expressed relative to the reference genes *Top1* and *Nono* and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤0.05, **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

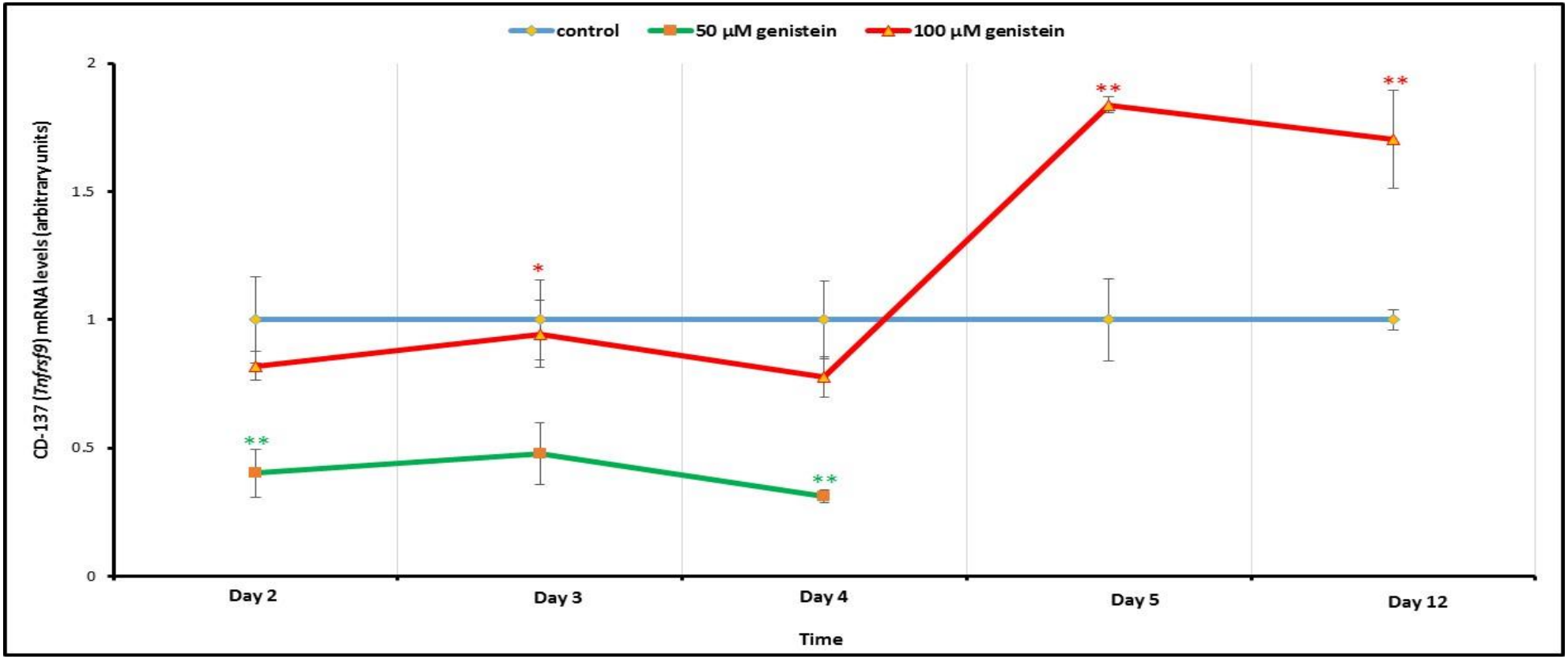


Figure 3. 16: The effect of genistein (10 µM, 50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to CD-137. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted. mRNA was measured by RT-qPCR. Data are expressed relative to the reference genes *Top1* and *Nono* and normalised to control. Values are means n=3-15 groups based on 3 technical replicates (wells from the same experiment) for 1-5 biological replicates (independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. *P≤0.05, **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

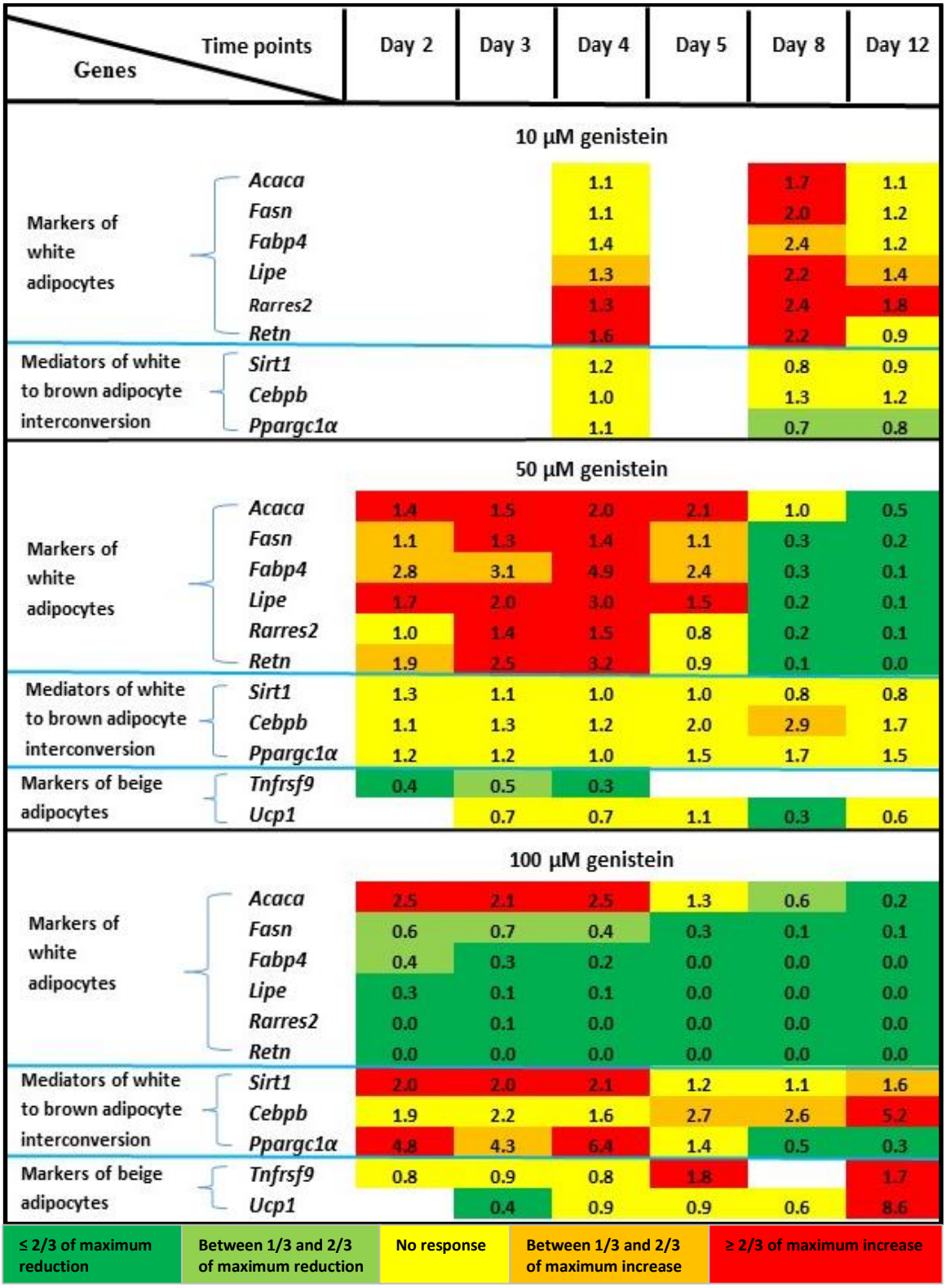


Figure 3. 17: A heat map to summarise data on effects of genistein at different concentrations as indicated on the expression of a panel of genes that are involved in lipogenesis (Acaca, Fasn), or white adipocyte maturation (Fabp4, Lipe, Rarres2, Retn), involved in mediating white to brown adipocyte interconversion or genes that characterise beige adipocytes (Ucp1, Tnfrsf9). Cells are coloured depending on the response size according to the key. For each gene the maximum increase or reduction in mRNA was determined and responses are shown as a portion. Numerical values normalised to control.

3.3.3. The effect of an ER antagonist on the responses of genes to genistein in 3T3-L1 cells

To test the hypothesis that the effects of genistein to apparently promote the conversion of white to beige adipocytes are a function of genistein binding to the ER 3T3-L1 preadipocytes were induced to differentiate in the presence and absence of genistein (50 µM and 100 µM) and fulvestrant (0.1 µM), which is a potent estrogen receptor antagonist with the ability to block both AF1 and AF2 domains of the estrogen receptor with no agonistic effects. On binding with the ER fulvestrant prevents receptor dimerization and translocation into the nucleus and promotes receptor degradation (Howell, 2006; Hutcheson *et al.*, 2011). The chemical structure of fulvestrant is shown in Figure 3.25.

Measurement of the panel of mRNAs corresponding to genes characteristic of white adipocytes (*Acaca, Fasn, Fabp4, Lipe, Rarres2 and Retn*) revealed that fulvestrant did not interfere with the effect of genistein to reduce expression of these genes indicating that these responses are an action of genistein that is independent of binding to the ER. Likewise, measurement of mRNAs corresponding to genes that mediate the conversion of white to beige adipocytes (*Sirt1, Ppargc1a*) and of *Ucp1*, which is characteristic of beige adipocytes, showed that fulvestrant did not attenuate the effect of genistein to decrease expression of these genes indicating that these responses are an action of genistein that is independent of binding to the ER (Figures 3.26 and 3.27).

An exception was C/EBPβ, for which fulvestrant reduced the size of the response to genistein consistent with this being a response mediated through the ER (Figure 3.28). Fulvestrant alone did not affect the mRNAs of this panel of genes.

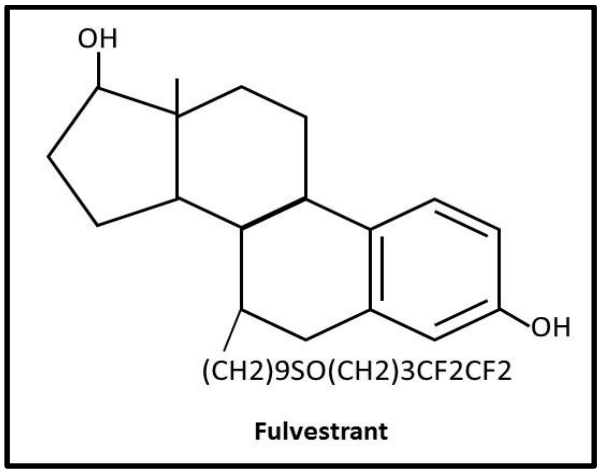


Figure 3. 18: Chemical structure of fulvestrant

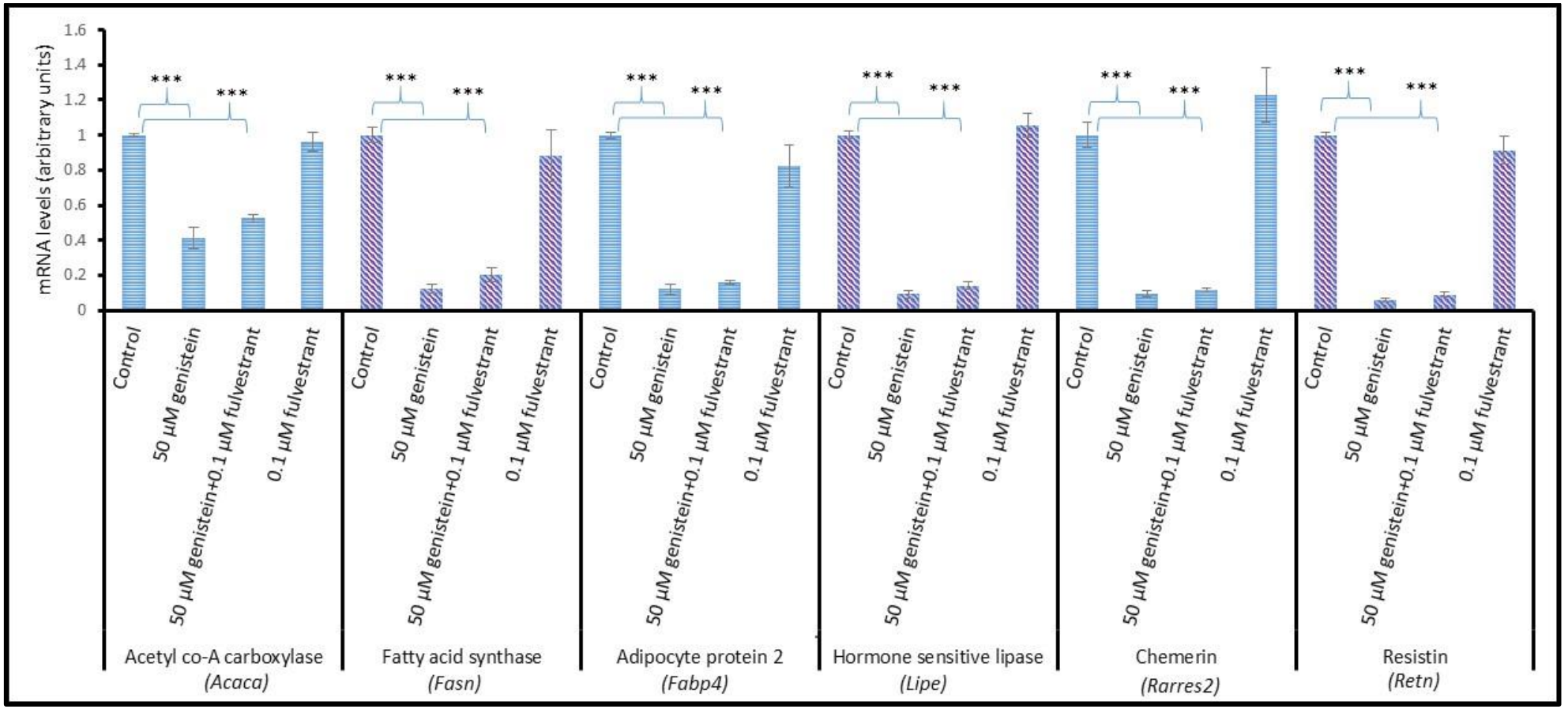


Figure 3. 19: The effect the ER antagonist fulvestrant (0.1 µM) on the response of mRNA corresponding to gene products involved in lipogenesis (ACC and FAS) or maturation of white adipocytes (AP2, HSL, chemerin and resistin) to 50 µM genistein applied on induction of differentiation of 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein and fulvestrant. Total RNA was extracted on day 12 and mRNA expression levels were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and NONO and normalised to control. Values are means (n= 3; three separate samples of one experiment), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

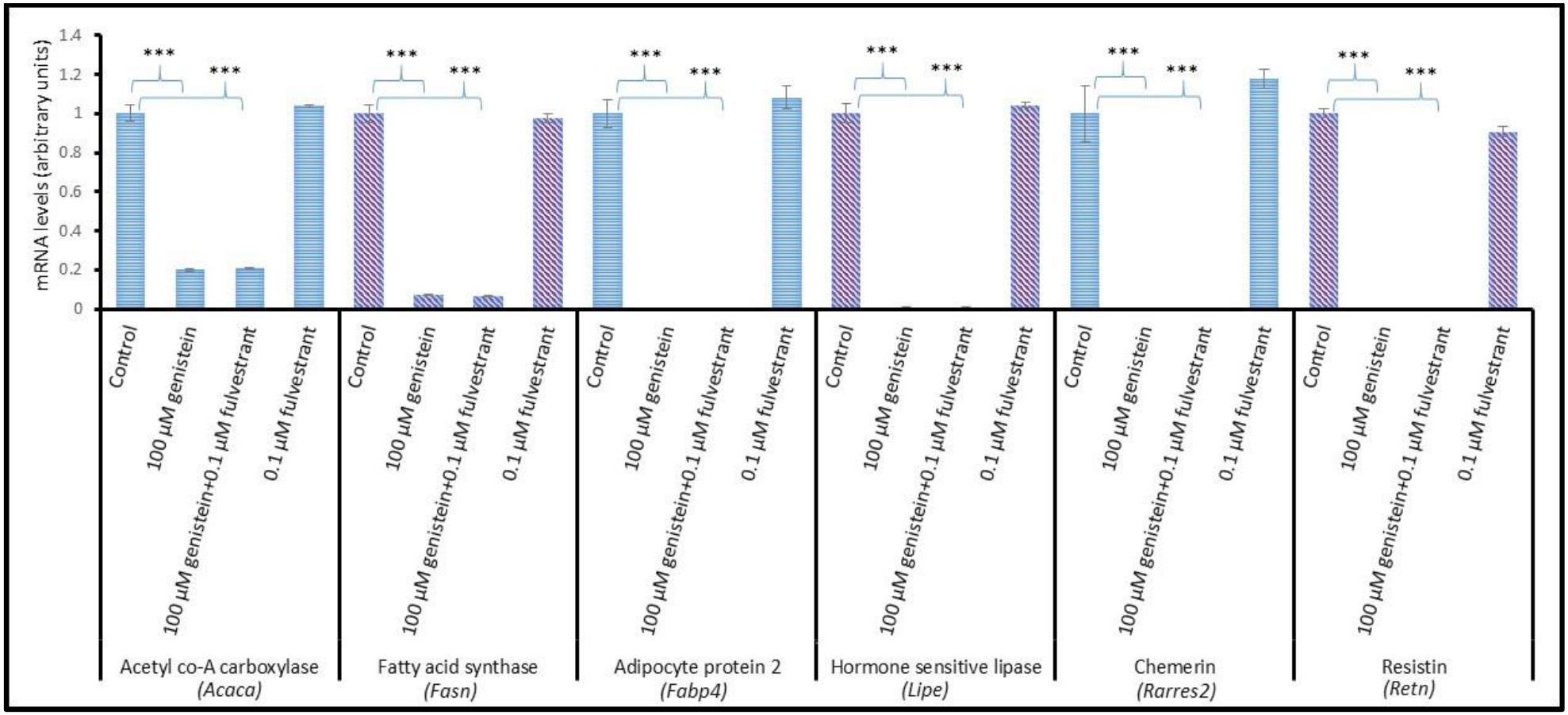


Figure 3. 20: The effect the ER antagonist 0.1 µM fulvestrant on the response of mRNA corresponding to gene products involved in lipogenesis (ACC and FAS) or maturation of white adipocytes (AP2, HSL, chemerin and resistin) to 100 µM genistein applied on induction of differentiation of 3T3-L1. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein and fulvestrant. Total RNA was extracted on day 12 and mRNAs expression levels were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and NONO and normalised to control. Values are means (n= 3; three separate samples of one experiment), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

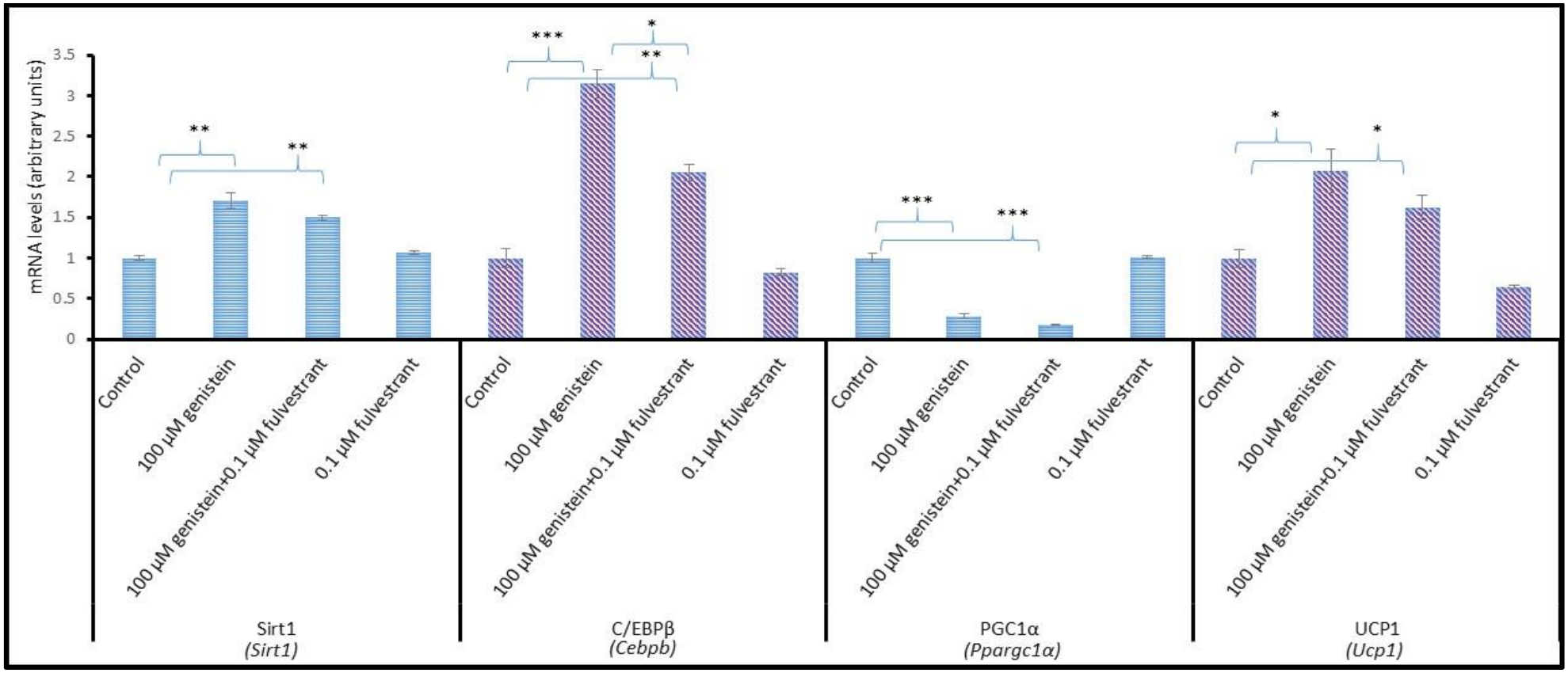


Figure 3. 21: The effect the ER antagonist 0.1 µM fulvestrant on the response of mRNA corresponding to gene products involved in interconversion of white to beige adipocytes (Sirt1, C/EBPβ and PGC1α) or marking beige adipocytes (UCP-1) to 100 µM genistein applied on induction of differentiation of 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein and fulvestrant on day 0 to day 12. Total RNA was extracted on day 12 and mRNA expression levels were measured by using RT-qPCR. Data are expressed relative to the reference genes Topo1 and NONO and normalised to control. Values are means (n= 3; three separate samples of one experiment), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001, **P≤0.01, *P≤0.05. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

3.3.3. The effect of a Sirt1 inhibitor on the responses of genes to genistein in 3T3-L1 cells

We hypothesised that Sirt1, which was increased in response to treatment of 3T3-L1 cells with the higher concentrations of genistein, was a mediator of the effect of genistein on other genes measured. To test this hypothesis cells were treated with genistein in the presence of Ex-527, which is a specific and potent Sirt1 inhibitor. Ex-527 has been seen to have low cytotoxic effects compared with other Sirt-1 inhibitors such as sirtinol, nicotinamide and salarmide (Napper *et al.*, 2005; Solomon *et al.*, 2006; Peck *et al.*, 2010). It appears to inhibit Sirt-1 by binding in the nicotinamide pocket of the Sirt-1 protein (Gertz *et al.*, 2013), and its inhibitory effects on Sirt1 activity have been confirmed in several cell models including rat H4IIEC3 hepatoma cells (Caton *et al.*, 2011), primary human mammary epithelial cells (HMEC), and MCF7 breast cancer cells (Solomon *et al.*, 2006). The chemical structure of EX-527 is shown in Figure 3.29.

3T3-L1 cells were induced to differentiate in the presence and absence of 100 µM genistein and 10 µM EX-527. Total RNA was extracted at day 12 and mRNA corresponding to the target genes was measured by RT-qPCR. The results showed that the genistein-stimulated down-regulation of genes that characterise white adipocytes (*Acaca, Fasn, Fabp4, Lipe, Rarres2 and Retn*) was not reversed in the presence of EX-527 (Figure 3.30). However, the expression of this panel of genes was increased significantly when the cells were induced to differentiate in the presence of EX-527 alone (Figure 3.30), which indicates the involvement of Sirt1 activity in regulating the process of lipogenesis. However, inhibition of Sirt1 activity reduced the genistein-mediated up-regulation of UCP1 and C/EBPβ but Sirt1 inhibition alone did not affect these mRNAs (Figure 3.31) revealing that at least a component of these responses is via Sirt1.

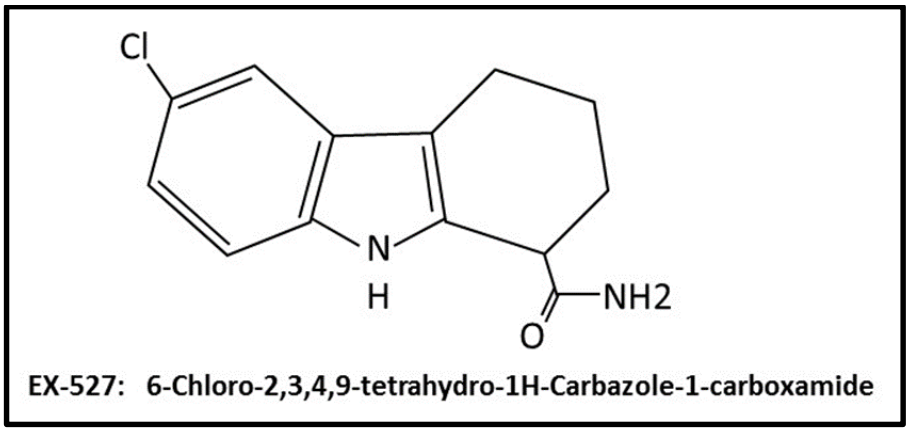


Figure 3. 22: Chemical structure of EX-527

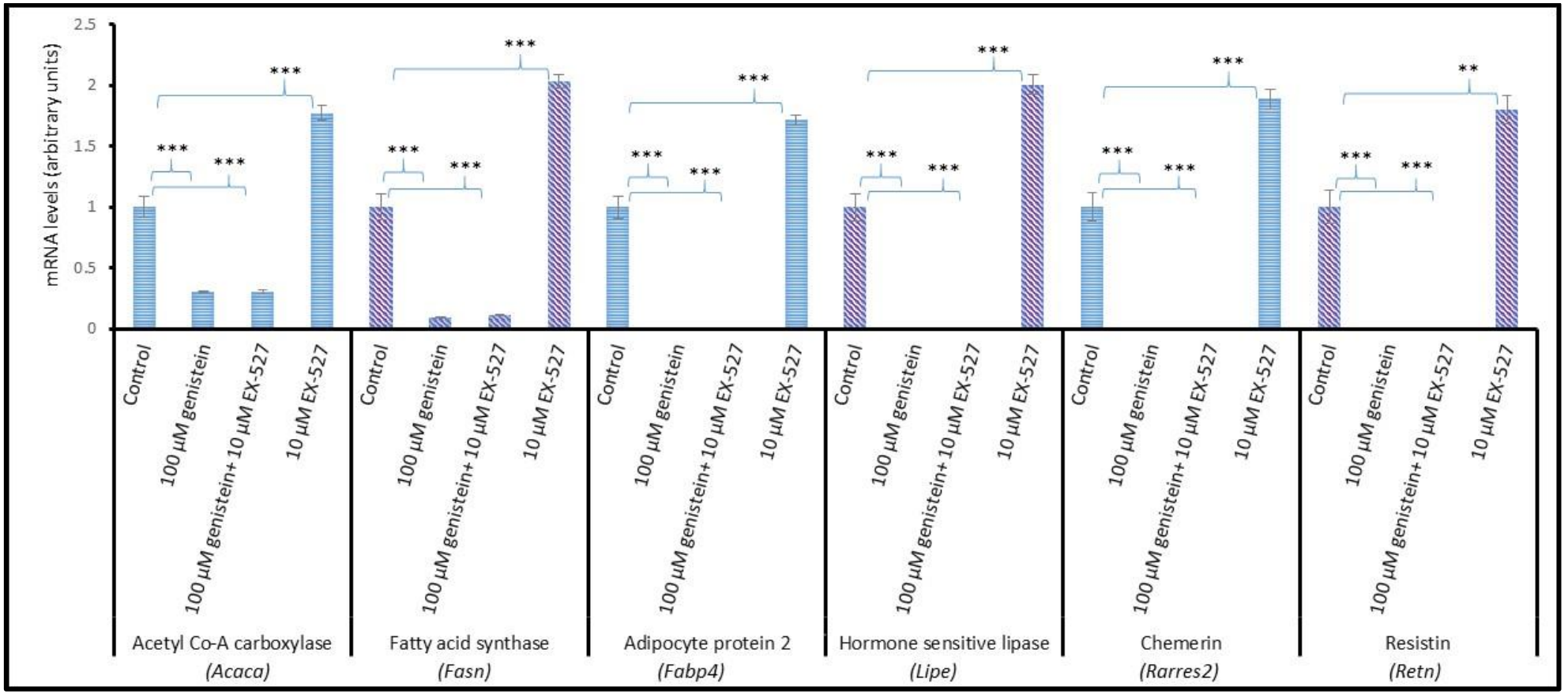


Figure 3. 23: The effect of Sirt1 inhibitor EX-527 (10 µM) on the response of mRNA corresponding to gene products involved in lipogenesis (ACC and FAS) or maturation of white adipocytes (AP2, HSL, chemerin and resistin) to 100 µM genistein applied on induction of differentiation of 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein EX-527. Total RNA was extracted on day 12 and mRNA expression levels were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and NONO and normalised to control. Values are means (n= 6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001, **P≤0.01. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

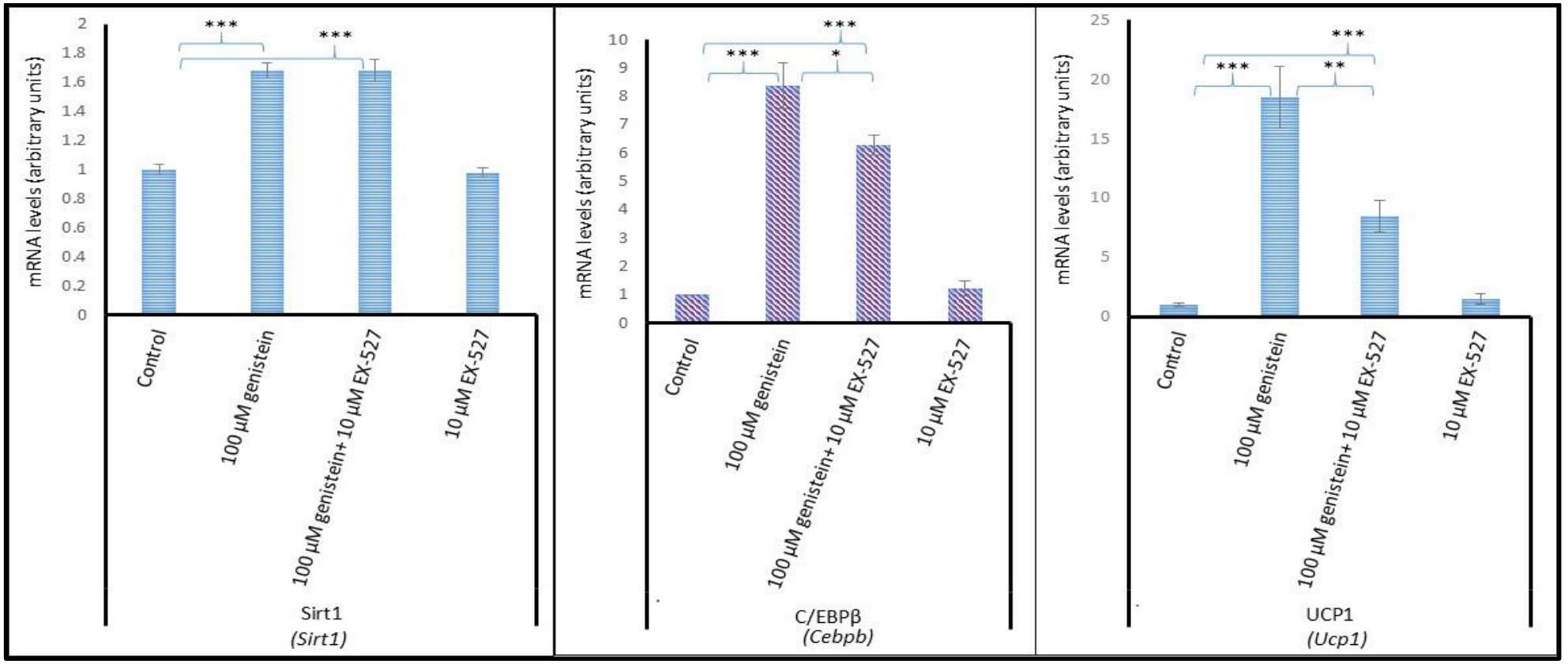


Figure 3. 24: The effect of Sirt1 inhibitor EX-527 (10 µM) on the response of mRNAs corresponding to gene products involved in white to beige adipocyte interconversion (Sirt1, C/EBPβ) or gene characterises brown adipocytes (UCP-1) to 100 µM genistein applied on induction of differentiation of 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. 48 h after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein and EX-527. Total RNA was extracted on day 12 and mRNA expression levels were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and NONO and normalised to control. Values are means (n= 6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001, **P≤0.01, *P≤0.05. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

3.3.4. The effect of genistein on mitochondrial function in 3T3-L1 cells

I determined if the observed effects of genistein on lipid accumulation and gene expression in 3T3-L1 cells that were consistent with a switch in phenotype from white to beige adipocytes were accompanied by the predicted uncoupling of mitochondrial respiration from oxygen consumption. To do this I measured oxygen consumption under conditions where action of the mitochondrial electron transport chain was manipulated following treatment of cells with genistein.

The seahorse XF-24 analyser was used for these experiments. After inducing the cells to differentiate in the presence and absence of genistein in Seahorse V7 cell plates, cellular respiration was measured at day 12. A baseline rate of oxygen consumption, including both oxidative phosphorylation and uncoupled oxygen consumption, was measured. Oxidative phosphorylation was then blocked using oligomycin (an ATP synthase inhibitor) (Symersky *et al.*, 2012). UCP1 activity was then stimulated using TTNBP (Figure 3.32) (Tomás et al., 2004) a retinoid (Mercadera et al., 2007) that induces thermogenesis through UCP1 activation (Rial *et al.*, 1999; Tomás *et al.*, 2004). The potent mitochondrial uncoupler FCCP was then added, allowing free movement of protons from intermembrane space into the mitochondrial matrix through inner mitochondrial membrane (Brennan *et al.*, 2006). Finally, oxygen consumption was blocked using antimycin A, which is a complex III inhibitor (Huang *et al.*, 2005).

Concomitant with the marked increase in UCP1 expression observed in response to genistein there was a significant increase in basal and uncoupled oxygen consumption rate compared with control (Figure 3.33A, 3.33B). In addition, the proportion of the total oxygen consumption that was uncoupled before FCCP addition and after activation of UCP1 by TTNBP was increased (Figure 3.33C). Together these measurements are consistent with a higher level of uncoupled oxygen consumption in cells treated with genistein consistent with the induction of a beige adipocyte phenotype.

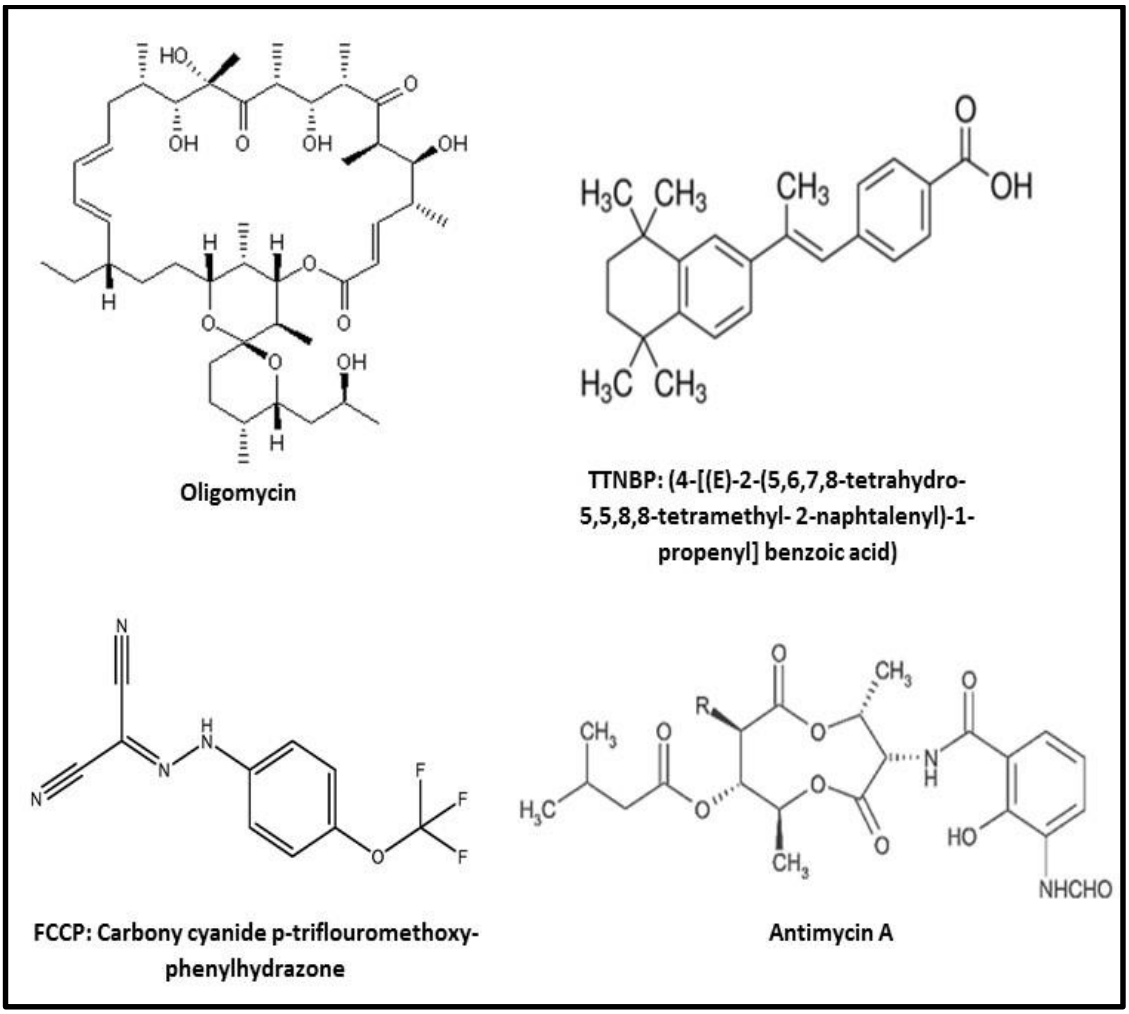


Figure 3. 25: Chemical structure of oligomycin, TTNBP, FCCP and antimycin A (https://www.sigmaaldrich.com/united-kingdom.html)

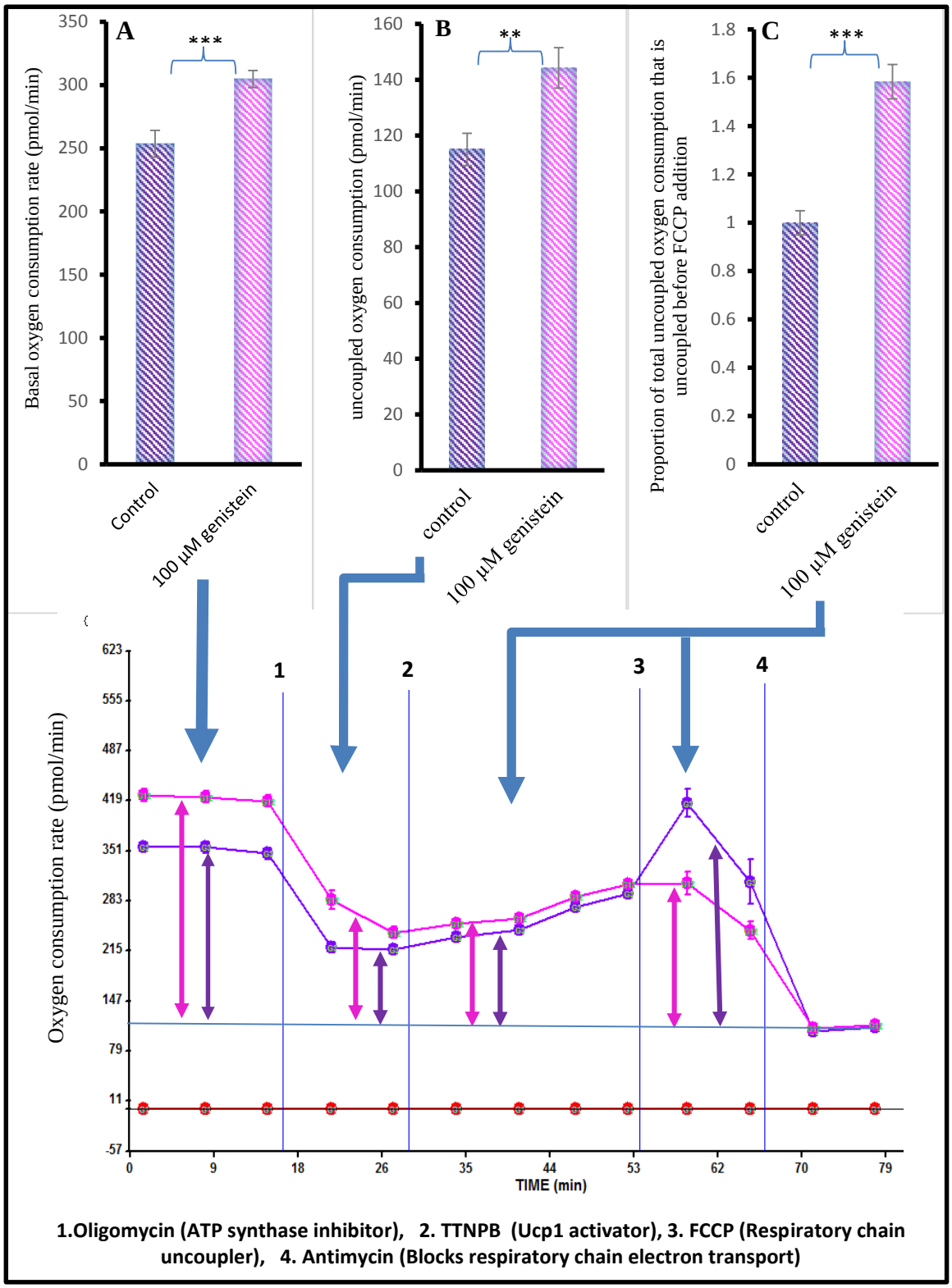


Figure 3. 26: The effect of genistein on mitochondrial function measured as oxygen consumption in 3T3-L1 maturing preadipocytes. Cells were cultured and grown to confluence Seahorse V7 cell plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein (100 µM). Oxygen consumption was measured on day 12 using Seahorse XF-24 analyser. Data are expressed relative to total protein, measured in the same samples. Values are means (n= 10), with standard errors represented by vertical bars. Statistical analysis was by unpaired Student`s T-test. ***P≤0.001.

3.3.5. The effect of genistein on Sirt1 protein abundance in 3T3-L1 cells

Sirt1 is a focus of other complementary research in the laboratory. Thus I investigated further the effect of genistein on Sirt1 in 3T3-L1 cells by determining how genistein affected the abundance of Sirt1 protein. 3T3-L1 cells were induced to differentiate in the presence and absence of 100 μM genistein then total protein was extracted at day 10 of differentiation and abundance of Sirt1 protein was measured by western blotting. The results showed that despite the increase in Sirt1 mRNA induced by genistein being only moderate Sirt1 protein abundance was increased by several fold (Figure 3.34).

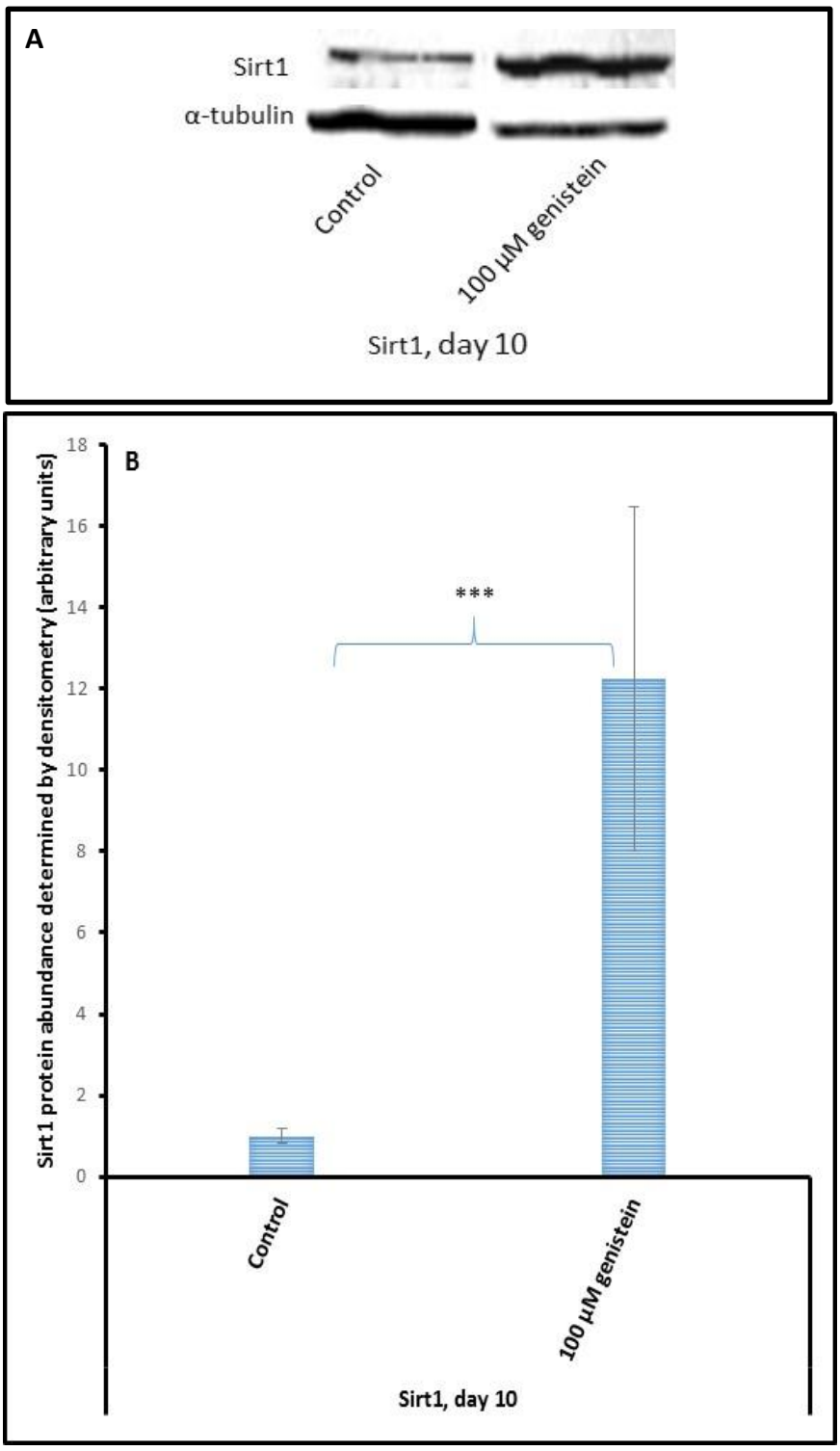


Figure 3. 27: The effect of 100 µM genistein applied on induction of differentiation on Sirt1 protein abundance in 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of 100 µM genistein. On day 10 total protein was extracted. Histone H3 expression was measured by western blotting. Panel A shows representative western blots. Bands were quantified using Odyssey densitometry software to generate the data shown in panel B. Data are expressed relative to the corresponding α-tubulin then normalised to control. Values are means (n=5; three and two separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. ***P≤0.001.

3.3.6. The effect of genistein on Sirt1 expression in other cell models

I investigated if the effect of genistein on Sirt1 expression is cell and/or species specific and also determined if the response is mediated through the ER or through the GPR30 receptor for estrogen action (Hutchens *et al.*, 2012; Luo *et al.*, 2014). To do this MCF-7 cells were treated with 100 µM genistein 24 h after seeding in the presence and absence of the ER antagonist fulvestrant (0.1 µM) or G-15 (1 µM) for 48 h. G-15 (1 µM) is a specific GPR30 antagonist (Dennis *et al.*, 2009; Prossnitz and Barton, 2011) and thus inhibits the GPR30 mediated estrogen response pathway (Hutchens *et al.*, 2012; Luo *et al.*, 2014).

As observed in 3T3-L1 cells Sirt1 mRNA measured by RT-qPCR (primer sequences are stated in chapter 2, table 2) was increased and neither ER antagonist affected the response to genistein (Figures 3.35 and 3.36), showing that the effect of genistein on Sirt1 is ER-independent and not cell type and/or species specific.

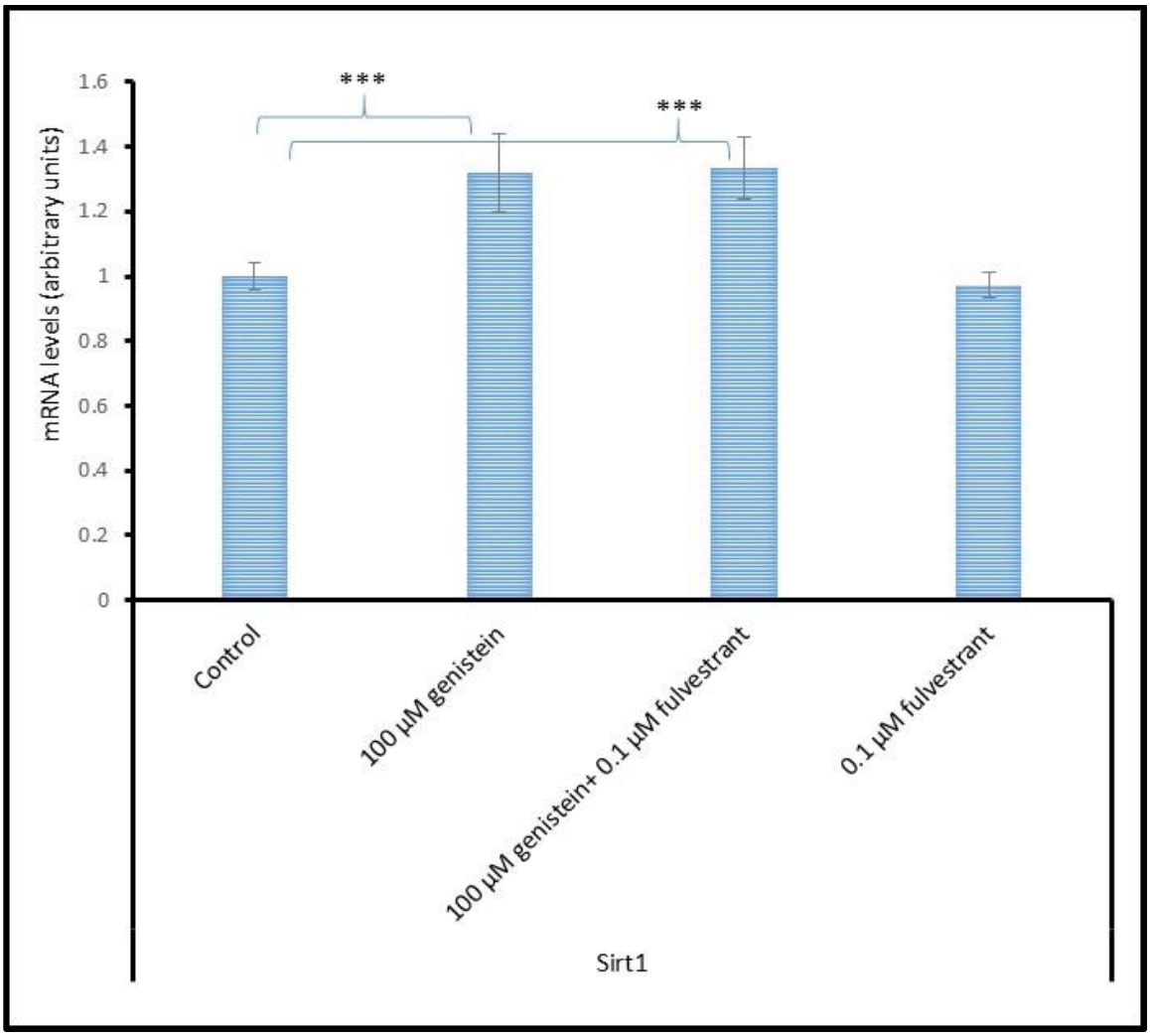


Figure 3. 28: The effect of the ER antagonist fulvestrant on the response of Sirt1 to 100 µM genistein in MCF-7 cells. Cells were cultured and grown in 6-well plates. Twenty four hours after seeding cells were treated with 100 µM genistein in the presence and absence of 0.1 µM fulvestrant for 48 h. Total RNA was extracted and Sirt1 mRNA level was measured by RT-qPCR. Data are expressed relative to the reference genes TOPO1 and GAPDH and normalised to control. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

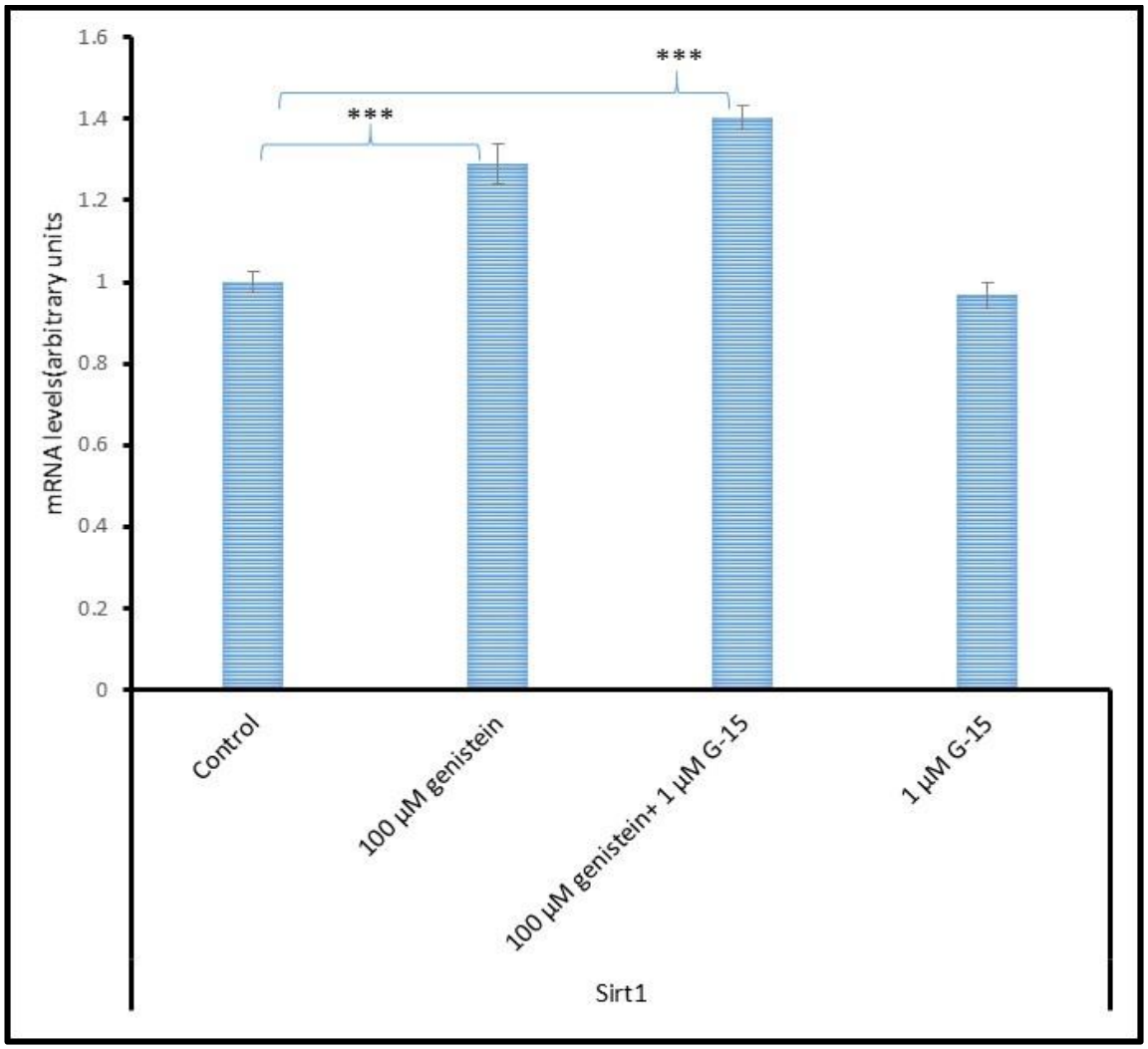


Figure 3. 29: The effect of the ER antagonist G-15 on the response of Sirt1 to 100 µM genistein in MCF-7 cells. Cells were cultured and grown in 6-well plates. Twenty four hours after seeding cells were treated with 100 µM genistein in the presence and absence of 1 µM G-15 for 48 h. Total RNA was extracted and Sirt1 mRNA level was measured by RT-qPCR. Data are expressed relative to the reference genes TOPO1 and GAPDH and normalised to control. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

3.3.7. The effect of genistein on the expression of reference genes in 3T3-L1 cells

The expression of reference genes sued as an internal control for RT-qPCR may vary considerably under experimental conditions to select suitable reference genes that were not affected by genistein I measured the effect of genistein in 3T3-L1 cells on the RNA correspondent to *Topo1*, *Nono* and *Gapdh*, which are commonly used as reference genes. For Top1 and Nono mean Ct value measured over 3 samples were within 0.2 cycles for control cells and cells exposed to 50 μM genistein using the standard experimental protocol and within 0.4 cycles for cells exposed to 100 μM genistein (Figure 3.31). However, the mean Ct value for GAPDH increased substantially at both concentration of genistein (>1.4 cycles at 50 μM and >1.8 cycles at 100 μM), indicating that the treatment caused a reduction in GAPDH in the order of 2.6 and 3.5 folds. Thus Nono and Topo1 were used as reference genes in all experiments involving treatment of 3T3-L1 cells.

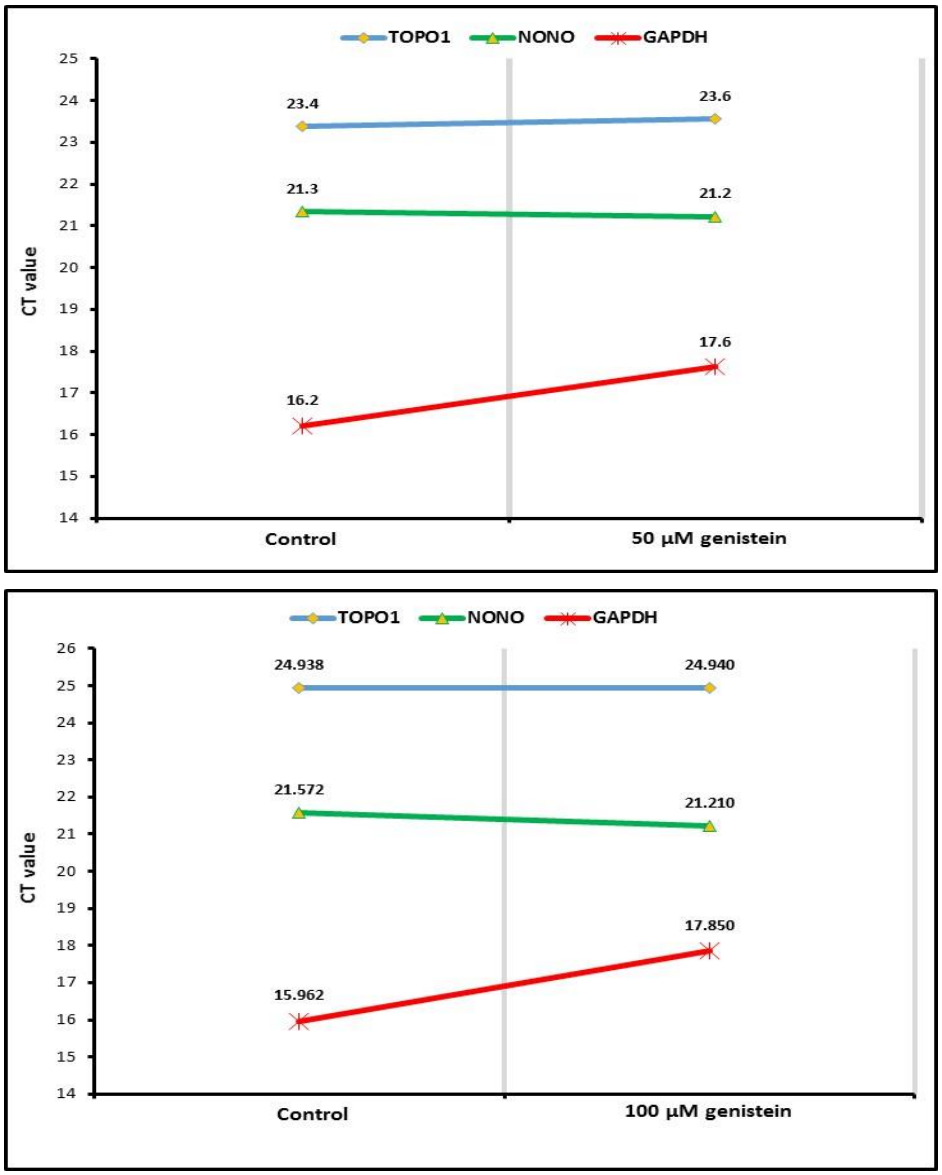


Figure 3. 30: The effect of genistein (50 µM or 100 µM) applied on induction of differentiation of 3T3-L1 cells on mRNA corresponding to TOPO1, NONO and GAPDH. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. Total RNA was extracted at day 12. mRNA was measured by RT-qPCR. Data are expressed as mean CT values for n=3 separate wells from the same experiment.

3.4. Discussion

The work presented in this chapter developed from the observation that treatment of 3T3-L1 preadipocyte cells with genistein induced a change in appearance suggestive of a beige rather than white adipocyte phenotype. This observation was made when performing experiments to determine if genistein had effects similar to the polyphenol resveratrol on the expression of genes involved in lipogenesis including *Acaca* and *Fasn* in 3T3-L1 cells. These effects of resveratrol had been observed in previous experiments in the laboratory. To determine if genistein promoted a beige adipocyte phenotype, the morphological changes of 3T3-L1 cells were monitored by staining cells with oil-red-O, which is a lipid specific stain. I also determined if there was a change in the expression of a panel of marker genes selected on the basis that they were involved in; (I) lipid biogenesis (*Acaca, Fasn*) or white adipocyte maturation (*Fabp4, Lipe, Rarres2, Retn*), which I predicted to be down-regulated, (II) involved in mediating white to brown adipocyte interconversion (Sirt1, Cebpb, Ppargc1α), which I expected to be upregulated, (III) genes that can be used as markers of beige adipocytes (*Ucp1, Tnfrsf9*), which I also predicted would be up-regulated. I also determined if the change in gene expression profile was accompanied by the predicted uncoupling of mitochondrial oxygen consumption from ATP synthesis.

In support of our hypothesis there was an accumulation of small multilocular lipid-droplets within the cytoplasm of genistein treated cells, which contrasted with the typical large fat droplets observed in the control differentiated cells. Consistent with this morphological change, there was a large reduction in the expression of genes that are expressed exclusively by white adipocytes (*Acaca, Fasn, Fabp4, Lipe, Rarres2 and Retn)*, which was associated with a marked increase in the expression of genes mediating white to brown interconversion (*Sirt1, Cebpb, Ppargc1a),* and an increase in the expression of those genes characterise brown adipocytes, specifically *Ucp1* and *Tnfrsf9*. Concomitant with a marked increase in UCP1 expression observed in response to genistein there was a dramatic increase in basal and uncoupled oxygen consumption rate. In addition, the proportion of the total oxygen consumption rate that was uncoupled before FCCP addition and after activation of UCP1 by TTNBP was markedly increased. Together these measurements are consistent with increased uncoupling of mitochondrial oxygen

consumption from ATP synthesis and thus with the induction of a beige adipocyte phenotype.

Previous studies in rodents have shown that genistein alone or as a crude extract with other polyphenols can have an effect on energy expenditure and UCP expression. For example, genistein (5000-50,000 μg/Kg per a day) was found to upregulate UCP1 expression in white adipose tissue depots in mice (Penza et al., 2006). Also the obesogenic effect of a high caloric diet in mice was prevented by genistein (0.2%, 0.4% genistein in the diet for 12 weeks) concomitant with an increase in the expression of PGC1α, mitochondrial medium chain acyl Co-A dehydrogenase, UCP2, and mitochondrial biogenesis (Lee et al., 2006). Dietary soya protein containing isoflavones given to rats for 14 d or 180 d reduced body weight and energy expenditure and increased UCP expression in brown and white adipose tissue (Takahashi and Ide, 2008; Torre-Villalvazo *et al.*, 2008). Similarly, soya protein alone and/or in combination with exercise in rats stimulated the expression of PGC1α and PPARα lipid content of the liver and epididymal adipose tissue (Morifuji et al., 2006). Higher UCP1 expression and oxygen consumption rate was measured in rodents fed an isoflavone rich plant extract (*Puerariae thomsonii*) or a soya based diet compared with the control group (Lephart *et al.*, 2004; Kamiya *et al.*, 2012). It has also been shown that other polyphenols, including diadzein and resveratrol can increase UCP1 expression and mitochondrial biogenesis. For example, diadzein increased UCP1 expression in brown adipose tissue and reduced body weight in dietary induced obese rats (Crespillo et al., 2011). Resveratrol increased UCP expression and mitochondrial biogenesis in rat brown adipose tissue and muscle (Alberdi et al., 2013).

To the best of our knowledge this study is the first in vitro study to show the modulatory effect of genistein on white to brown adipocyte interconversion in 3T3-L1 cells. The establishment of this in vitro model had provided a valuable research tool with which to probe in detail mechanisms of diet induced white to beige adipocyte interconversion at a level of molecular detail that cannot be readily achieved using in vitro model. In addition, this observation indicates that the parent compound genistein rather than its metabolites is the active stimulator of this response. Here a caveat is that to draw such a conclusion unequivocally I would need to show that genistein is not metabolised in this cell culture system. The model thus also provide the opportunity to test individual

metabolite of genistein, as well as other dietary agents and their metabolite for activity. Our observations also confirm the fact that beige adipocytes have the same precursor as white adipocytes, which is distinct from that of classical brown adipocytes.

The fact that effects observed at the higher concentrations of genistein used (50 and 100 μM) mirrored effects also achieved at a lower concentrations (10 μM), but occurred over a shorter time scale, indicates that the responses to the higher concentrations were not the result of toxic action. The expectation would be that toxic effects would be distinct. However, I also saw a more direct indication that the higher concentrations of genistein were not toxic to the cells in that there was no decline in RNA yield as reported in section 2.4.2.2.

Since genistein resembles 17β-estradiol structurally and is, therefore, described as a phytoestrogen I determined if the responses I observed in 3T3-L1 cells were mediated through the ER. In the presence of the ER antagonist fulvestrant the observed change in cell appearance induced by genistein was not reversed and the changes in expression of our test gene panel was not abolished, with the exception of the response of *Cebpb* to genistein, which was significantly attenuated by fulvestrant. Thus the effect of genistein I observed appears to be not mediated via the ERs. A caveat to this conclusion is that I were unable to identify a suitable marker gene to measure as a positive control to confirm that the fulvestrant applied was effective in blocking all ER-mediated action. However, the attenuated response of *Cebpb* to genistein by fulvestrant is evidence of activity. The concentration of fulvestrant (0.1 μM) used has been shown in other work to be effective (Howell, 2006; Hutcheson *et al.*, 2011), and in parallel work (chapter 5) I observed an effect of the same concentration of fulvestrant on ERs in MCF-7 cells (see section 5.3.2). The ER-independent effects of genistein at the concentration I used are consistent with previous studies that have shown dose-dependent effects of genistein with respect to action at the ER or through other pathways, Lower concentrations of genistein (up to 3.7 μM) appear to have estrogen-like effects (Chen and Donovan, 2004; Magee and Rowland, 2004; Pons *et al.*, 2014) in different cell models including MCF-7 breast cancer cells, human cervical epithelial cells (HeLa), Caco-2 human intestinal cells, murine preosteoblastic cells (KS483) and mouse bone marrow cells (Maggiolini et al., 2001; Dang et al., 2003; Chen and Donovan, 2004). The action of genistein at higher concentrations,

however, appears to be via other cellular pathways (Dang et al., 2003). These actions measured at higher concentrations include inhibition of tyrosine kinase and topoisomerase activities (Salti et al., 2000; Chen and Donovan, 2004; Kasai and Kikuchi, 2010). Whether there are other pathways are conduits for genistein action to affect the response I observed requires further investigation.

I proposed that key initiating steps in the process of beige adipocyte differentiation induced by genistein would be reflected as changes in gene expression that occurred early in the sequence of effects I observed. I also proposed that genes reported to play an active role in the process of differentiation, as opposed to bystander marker genes, were prime candidates as pivotal initiating mediators. Our gene panel, Sirt1 met these criteria. Moreover, Sirt1 is a focus of other complementary research in the laboratory. Thus, I investigated if Sirt1 was necessary for genistein to affect the changes observed. In support of this hypothesis, the responses of *Ucp1* and *Cebpb* genes to genistein were significantly attenuated when the activity of Sirt1 was inhibited by EX-527, which is a specific and potent Sirt1 inhibitor with the ability to bind in the nicotinamide pocket of the Sirt-1 protein (Gertz *et al.*, 2013). The increase in Sirt1 mRNA I observed in response to treatment of 3T3-L1 cells with genistein was very moderate (2 fold). However, I measured an increase of approximately 12 fold in Sirt1 protein providing a further indication that increased Sirt1 action is a key mediator in the change I observed.

I also showed that the effect of genistein on Sirt1 expression was shown to not be unique to adipocytes. Genistein also was found to promote an increase in the expression of Sirt1 in MCF-7 breast cancer cells and the effect was ERs-independent similar to the observations made in 3T3-L1 cells. The stimulatory effect of genistein on Sirt1 expression may be one of the reasons behind the pleiotropic effects of genistein. Sirt1 is a class III histone deacetylase but has a diverse substrate range. Sirt1 thus affects fundamental processes including inflammatory and metabolic pathways (Revollo and Li, 2013; Davenport et al., 2014). Sirt1 has been proposed as a mediator of the lifespan extension observed in response to dietary restriction, consistent with observations such as extended life expectancy when pharmacologically activated in mice (Mitchell et al., 2014) and a decrease with age in the liver of old mice (Jin et al., 2011). Pharmacological activation of Sirt1 has been reported to have several health-beneficial actions including improvement

in the blood lipid profile and decreasing the risk of non-alcoholic fatty liver (Mitchell et al., 2014) and diabetes (Qiao and Shao, 2006).

It has been shown that Sirt1 has a key regulatory role especially in mediating beige adipocyte development. An increase in Sirt1 expression or its activation by resveratrol was followed by an increase in the expression of genes that characterise brown adipocytes including UCP1 and C/EBPβ in white adipose tissue depots of transgenic mice and 3T3-L1 cells, while there was a marked reduction in the expression of genes that characterise white adipocyte cells including *Rarres2* and *Retn* (Qiang *et al.*, 2012; Fu *et al.*, 2014). However, in classical brown adipocytes the function of Sirt1 appears to not be important in the regulation of UCP1 and C/EBPβ expression as an increase in Sirt1 expression or its knock-down in transgenic mice was found to have no effect on UCP1 and C/EBPβ expression (Qiang et al., 2012).

The increase in C/EBPβ expression induced by genistein in the present study is also likely to be important in the process of beige adipocyte development in response to genistein. C/EBPβ has emerged as a conspicuous player in the development of classical brown and beige adipocytes (Qiang et al., 2012). Expression of C/EBPβ appears to be crucial for differentiation of brown adipose tissue in mice (Tanaka *et al.*, 1997). Furthermore, overexpression of C/EBPβ in human skin fibroblasts, mouse skin fibroblasts and 3T3-L1 cells was reported to be sufficient to convert these cells into brown adipocytes (Karamanlidis et al., 2007; Kajimura et al., 2009).

The observed large increase in PGC1α expression by genistein, especially during the first few days of the treatment, is also likely to be important in the process of white to brown adipocyte interconversion. PGC1α has been recognised as a key stimulating factor for mitochondrial biogenesis, which occurs at a high level during the development of brown adipocytes (Rodgers et al., 2005; Guarente, 2007; Fernandez-Marcos and Auwerx, 2011). Also a strong correlation between PGC1α and Sirt1 activity has been suggested, activation of PGC1α by Sirt1 via deacetylation appears to be important in modulating white to brown adipocyte interconversion (Aquilano *et al.*, 2010; Fu *et al.*, 2014).

In conclusion the work presented in this chapter shows for the first time in a malleable in vitro adipocyte model that genistein can mediate the process of white to

beige adipocyte conversion. Important conclusions I draw from the finding are that beige adipocytes can differentiate from the same cell lineage as white adipocytes. However, further work is required to determine if the process of interconversion is transdifferentiation of mature white adipocytes or denovo differentiation of precursor cells induced by genistein. The in vitro system affords the opportunity to address this fundamental question through approaches such as genetically marking the mature white adipocytes (for example, with β-galactosidase under the control of white-adipocyte-specific promoter) thus observing the distribution of marked cells after induction of the phenotypic change by genistein. Another important outcome of the presented study is the role of Sirt1 in mediating white to brown interconversion. A better understanding of this process may allow ultimately for the development of new dietary or pharmacological strategies to promote beige adipocyte tissue development and thus protect against metabolic diseases.

Chapter 4

4. Effects of genistein and resveratrol on histone expression

4.1. Introduction

Histone proteins and their posttranslational modifications can be important in modulating life expectancy (Feser *et al.*, 2010b; Khan and Khan, 2010). There is a view supporting the idea that abundance of histone proteins can positively influence life expectancy (O'Sullivan et al., 2010). Supporting evidence includes that overexpression of histone proteins in yeast increased lifespan (Feser *et al.*, 2010b) and decreased global histone protein expression and/or loss of histone proteins at specific regions of the genome was associated with genome instability and aging in yeast and in human foetal lung fibroblast cells (IMR90) (Dang et al., 2009; O'Sullivan et al., 2010; Hu et al., 2014). The expression of histone H3 and H4 was lower in fibroblasts of old versus young human individual, and at late passage (P75) compared with early passage (P30) of fibroblasts (O'Sullivan *et al.*, 2010). Also the ratio of histone variants including H2A and H3 variants was found to be reduced compared with total histone expression level during aging in human lung embryonic fibroblast cells (Rogakou and Sekeri–Pataryas, 1999).

Specific histone protein variants appear to be important in gene regulation. For example, presence of histone H3.3 and/or its post-translational modifications, particularly at specific gene promoter and enhancer regions, was associated with transcriptional activity (Ahmad and Henikoff, 2002; Hake and Allis, 2006; Chen *et al.*, 2013a). Expression of this variant was found to be crucial for DNA replication during the S-phase of the cell division (Frey et al., 2014) and to be associated with the transcriptionally active sites of the genome in the chicken erythroid cell models HD24 and 6C2 (Jin and Felsenfeld, 2006). Abundance of this variant marks highly active and fully differentiated cells such as nerve cells (Das and Tyler, 2013). Histone H3.3 knockdown in mouse resulted in abnormal chromosomal segregation (Bush et al., 2013) and defective decondensation of the pericentromeric chromatin (Lin *et al.*, 2013). Histone H3.2, on the other hand, appears to be associated preferentially with silenced loci and with heterochromatin formation, whereas histone H3.1 seems to have bidirectional (activation and/or silencing) effects on transcription (Hake and Allis, 2006). Replacement of histone H3.3 with histone H3.1

prevented C2C12 differentiation into myocytes and prevented lineage specific gene expression (Harada et al., 2014). Also, histones H3.1 and H3.2 were found to be associated with heterochromatin formation during fertilization and embryogenesis in the mouse (Akiyama et al., 2011).

4.2. Objective

Initial objective of this study was to replicate experiments to attempt to confirm observations made previously in the laboratory (Alatawi, 2012). These observations were a reduction in the level of histone protein abundance and in the activity of a histone H3.1 (variant H3b)-promoter reporter plasmid construct induced in Caco-2 human intestinal cells by 10 μM resveratrol. In the earlier work these effects were abolished in the presence of the ER-antagonist fulvestrant, indicating that this action of resveratrol was via the ERs. Thus, a subsidiary aim was to confirm this effect. A second aim was to investigate if genistein produced similar effects and also to determine if genistein modifies (either increases or reduces) the response to resveratrol. The rationale for this approach was that genistein and resveratrol are both polyphenols and have both been shown to have actions mediated via ERs. Secondly, in the natural diet, resveratrol and genistein can occur together, hence their interacting effects are worthy of investigation.

4.3. Results

4.3.1. The effect of genistein and resveratrol on a histone H3.1 promoter-reporter plasmid construct

To repeat and attempt to replicate earlier work in the laboratory, Caco-2 cells were transfected with a plasmid comprising the region -18 to -826 relative to the transcription start site of the human histone H3.1 (variant H3b) gene upstream of a β-galactosidase reporter gene. The transfected cells were then treated with 10 μM resveratrol in either the presence or absence of the ER antagonist fulvestrant (0.1 μM). In agreement with the earlier observations, lower levels of β-galactosidase activity in extracts from transfected Caco-2 cells were observed after treatment of cells with resveratrol compared with controls. However, in contrast to the earlier work, the response was not affected by fulvestrant (Figure 4.1).

Subsequent experiments investigated if genistein affected expression of the reporter gene from the same construct in Caco-2 cells. The effect observed was similar to that seen with resveratrol and also was not affected by fulvestrant (Figure 4.2). These data indicate that both resveratrol and genistein act on the human histone H3.1 (variant H3b) promoter to repress transcription and that the mechanism of action is independent of ERs. I also investigated the effect of resveratrol and genistein in combination on histone H3 gene transcription using the same experimented model. Reduced reporter gene expression was observed after treatment with the combined compounds but the effect was not greater than that observed with either compound separately. Also, as predicted based on the lack of effect on the responses to the single compounds, this response was not affected by fulvestrant (Figure 4.3). These data indicate that resveratrol and genistein act through the same ER-independent mechanism to affect histone H3.1 (variant H3b) promoter activity.

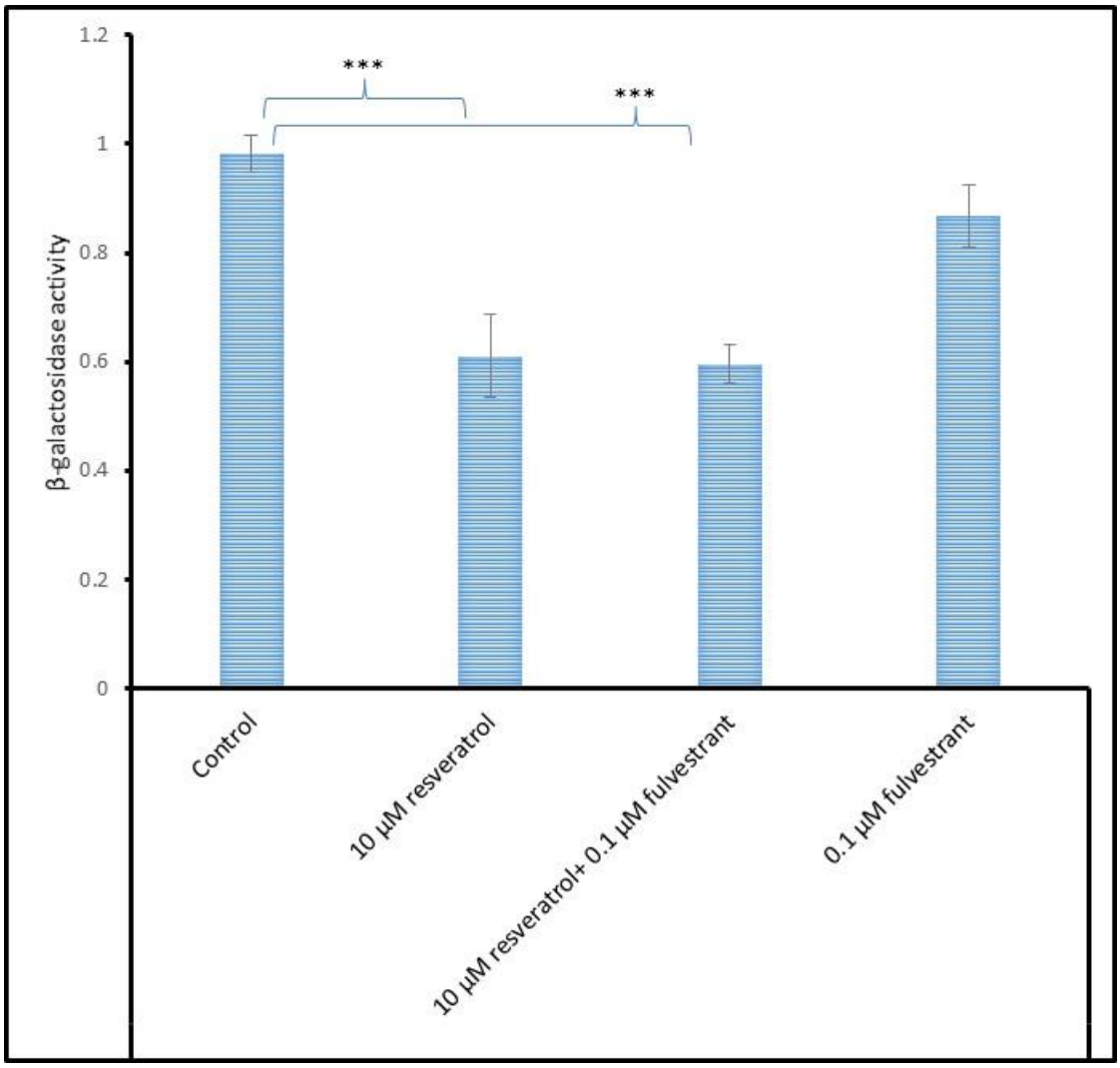


Figure 4. 1: The effect of resveratrol in the presence and absence of the ER antagonist fulvestrant (0.1 µM) on the response of a human histone H3.1 (variant H3b) promoter reporter plasmid to resveratrol. Data are derived from Caco-2 cells transfected with a histone H3.1 promoter-reporter-construct comprising the region -18 to -826 relative to the transcription start site of the human histone H3.1 (variant H3b) gene. Cells were treated with 10 µM resveratrol with or without 0.1 µM fulvestrant 24 h after transfection and reporter gene activity was measured after further 48 h. Data are expressed relative to total protein and normalised to control values. Values are means n=12-21, with standard errors are represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001.

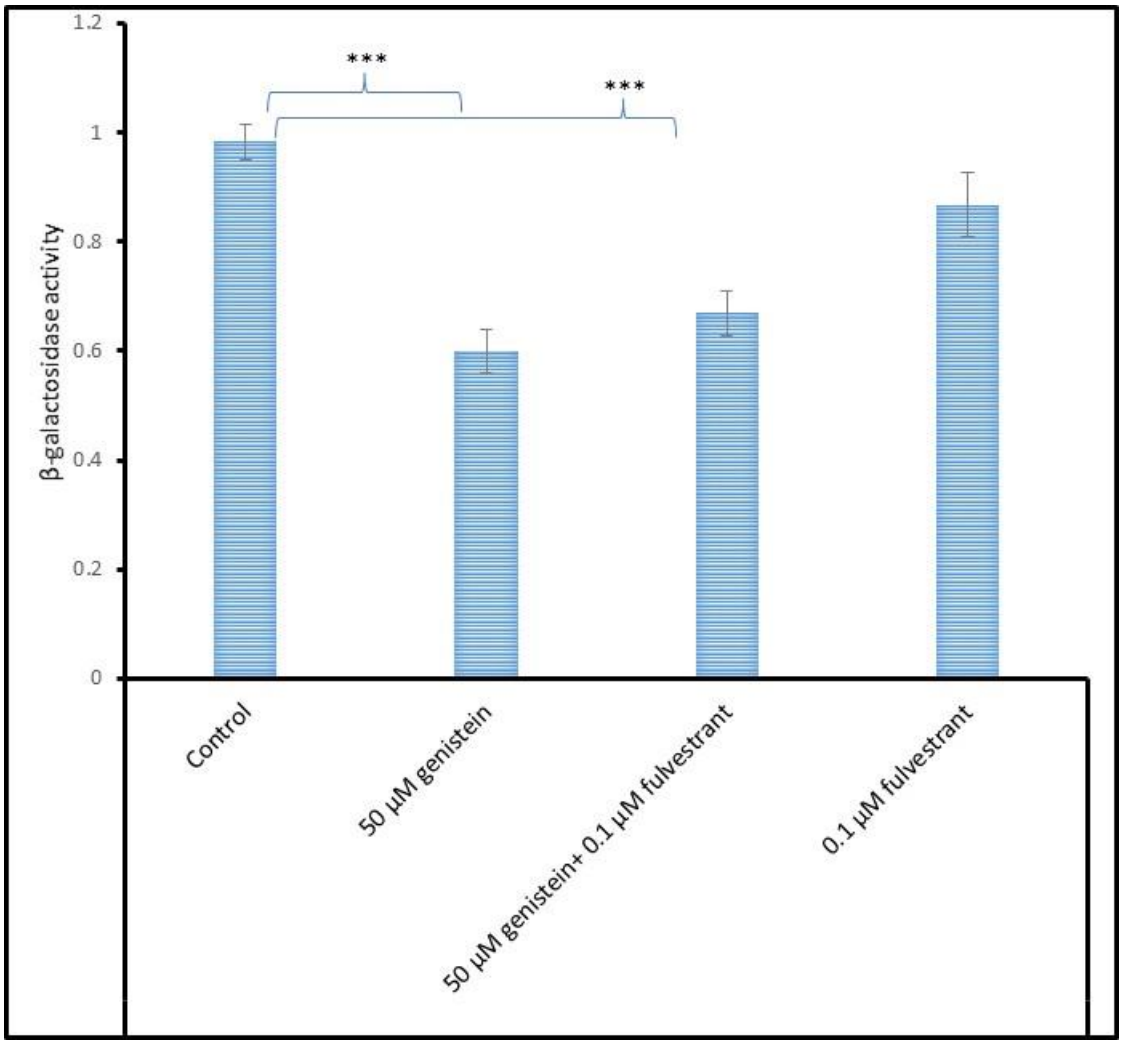


Figure 4. 2: The effect of genistein in the presence and absence of the ER antagonist fulvestrant on the response of a human histone H3.1 (variant H3b) promoter reporter plasmid to resveratrol. Data are derived from Caco-2 cells transfected with a histone H3.1 promoter-reporter-construct comprising the region -18 to -826 relative to the transcription start site of the human histone H3.1 (variant H3b) gene. Cells were treated with 50 µM genistein with or without 0.1 µM fulvestrant 24 h after transfection and reporter gene activity was measured after further 48 h. Data are expressed relative to total protein and normalised to control values. Values are means (n=12-21), with standard errors are represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001.

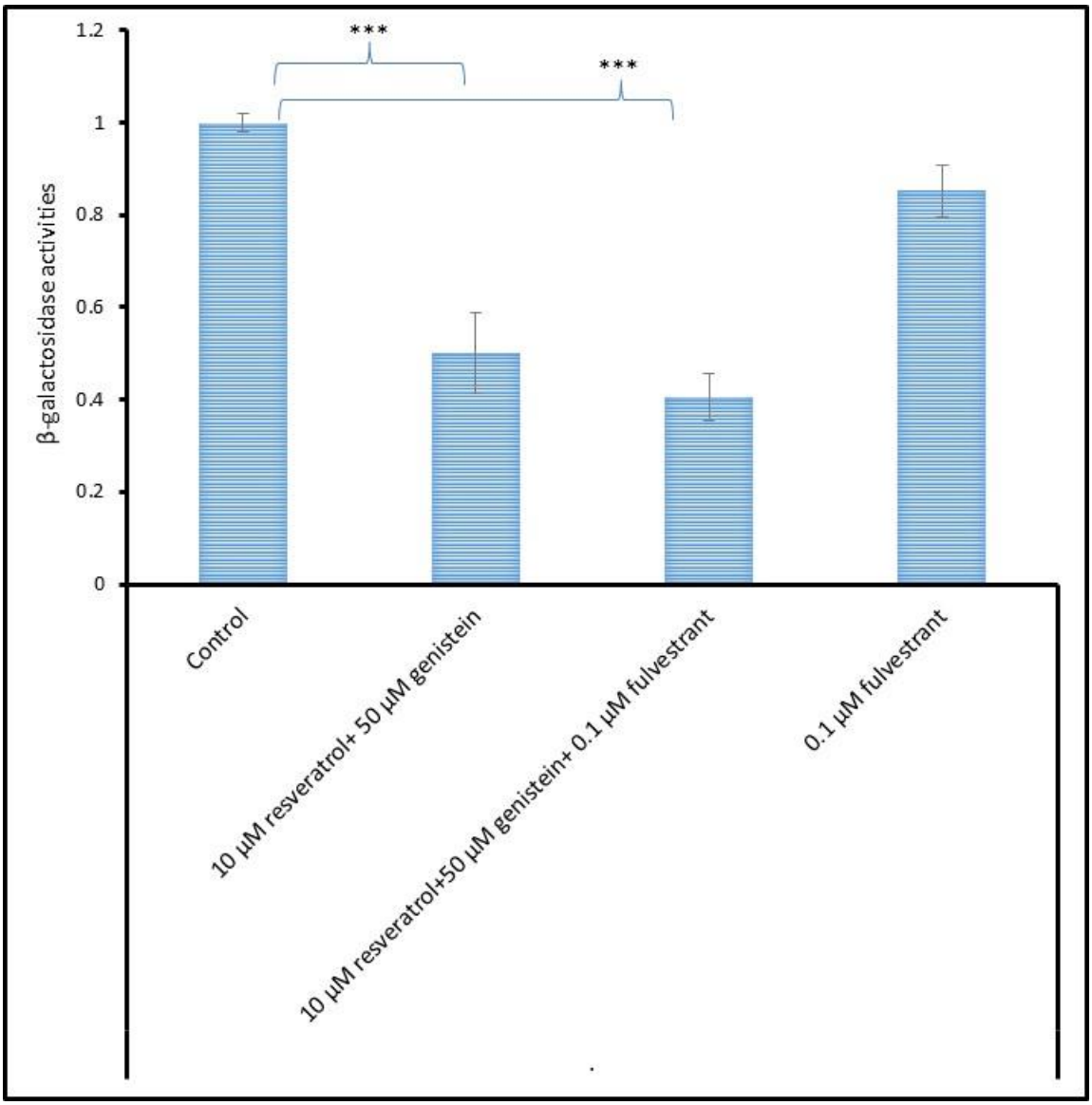


Figure 4. 3: The effect of genistein and resveratrol in the presence and absence of the ER antagonist fulvestrant on the response of a human histone H3.1 (variant H3b) promoter reporter plasmid to resveratrol. Data are derived from Caco-2 cells transfected with a histone H3.1 promoter-reporter-construct comprising the region -18 to -826 relative to the transcription start site of the human histone H3.1 (variant H3b) gene. Cells were treated with 50 µM genistein and 10µM resveratrol with or without 0.1 µM fulvestrant 24 h after transfection and reporter gene activity was measured after further 48 h. Data are expressed relative to total protein and normalised to control values. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors are represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001.

4.3.2. The effect of genistein on histone protein abundance

The reduced expression of the β-galactosidase reporter gene driven by the human histone H3.1 (variant H3b) promoter observed after treatment with resveratrol and genistein in combination was not greater than that observed with either compound separately. Hence further experiments focused on the effect of genistein alone, since this was the polyphenol of primary interest in the current work. The effect of genistein on histone protein abundance was measured in different cell models-MCF-7 (human breast cancer), Caco-2 (human intestinal) and 3T3-L1 (mouse preadipocytes) to determine if the action of genistein on the histone H3.1 (Variant H3b) promoter in Caco-2 cells was manifest as a reduction in abundance of the corresponding protein and if a similar effect was seen in other cell types.

In contrast to the predicted effect, genistein (100 μM) increased histone H3 and histone H4 protein in both Caco-2 and MCF-7 cell lines as measured by western blotting (Figures 4.4 and 4.5). I also measured the effect of genistein on histone H2B in MCF-7 cells and saw no change (Figure 4.6). Also, similar to the observations made in Caco2 and MCF-7 cells, histone H3 protein in 3T3-L1 cells increased in response to genistein applied on induction of differentiation (Figure 4.7).

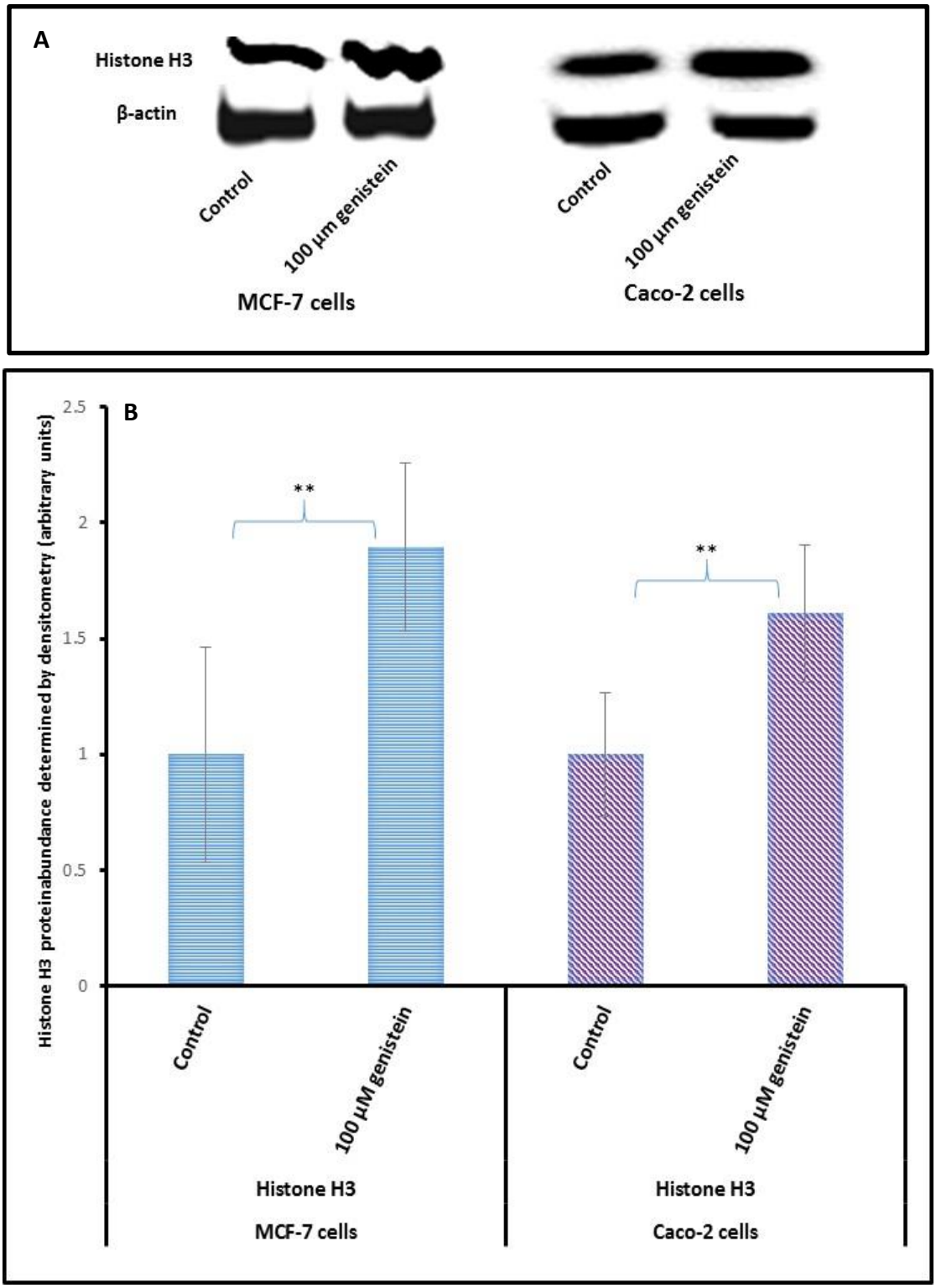


Figure 4. 4: The effect of genistein on histone H3 protein abundance in MCF-7 and Caco-2 cells. Forty eight hours after treatment total protein was extracted and histone H3 protein abundance was measured by western blotting. Panel A shows a typical representative western blot. Bands were quantified using Odyssey densitometry software to generate the data shown in panel B. Data are expressed relative to β-actin and normalised to control. Values are means (n=6; three separate wells for each of two independent experiments), with standard deviations are represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. **P≤0.01.

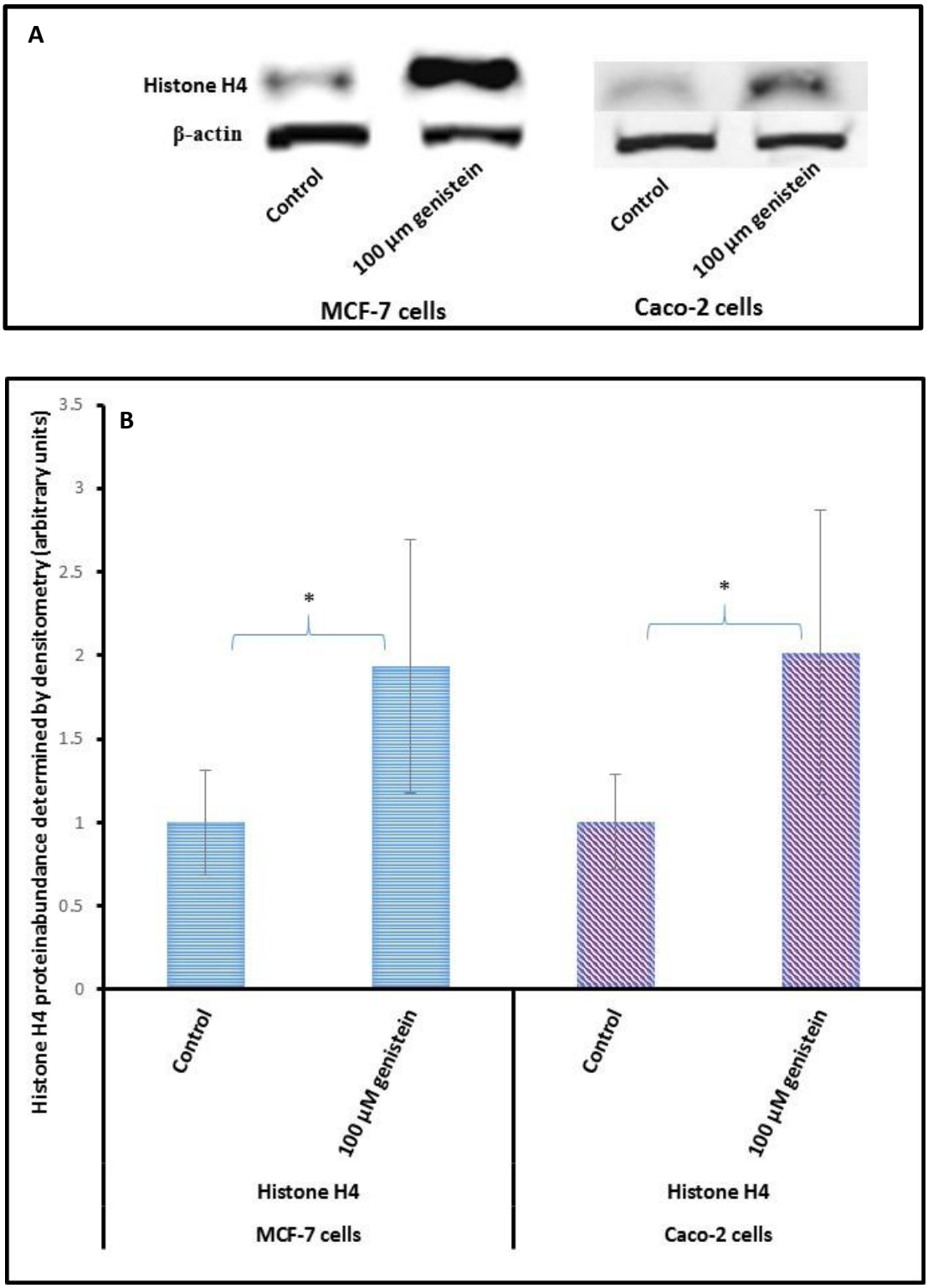
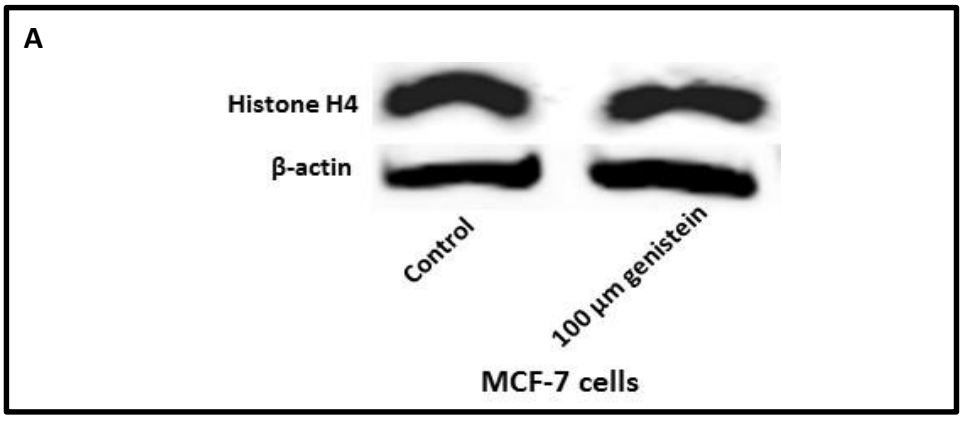


Figure 4. 5: The effect of genistein on histone H4 protein abundance in MCF-7 and Caco-2 cells. Forty eight hours after treatment total protein was extracted and histone H4 protein abundance was measured by western blotting. Panel A shows a typical representative western blot. Bands were quantified using Odyssey densitometry software to generate the data shown in panel B. Data are expressed relative to β-actin and normalised to control values. Values are means (n=6; three separate wells for each of two independent experiments), with standard deviations are represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. *P≤0.05.



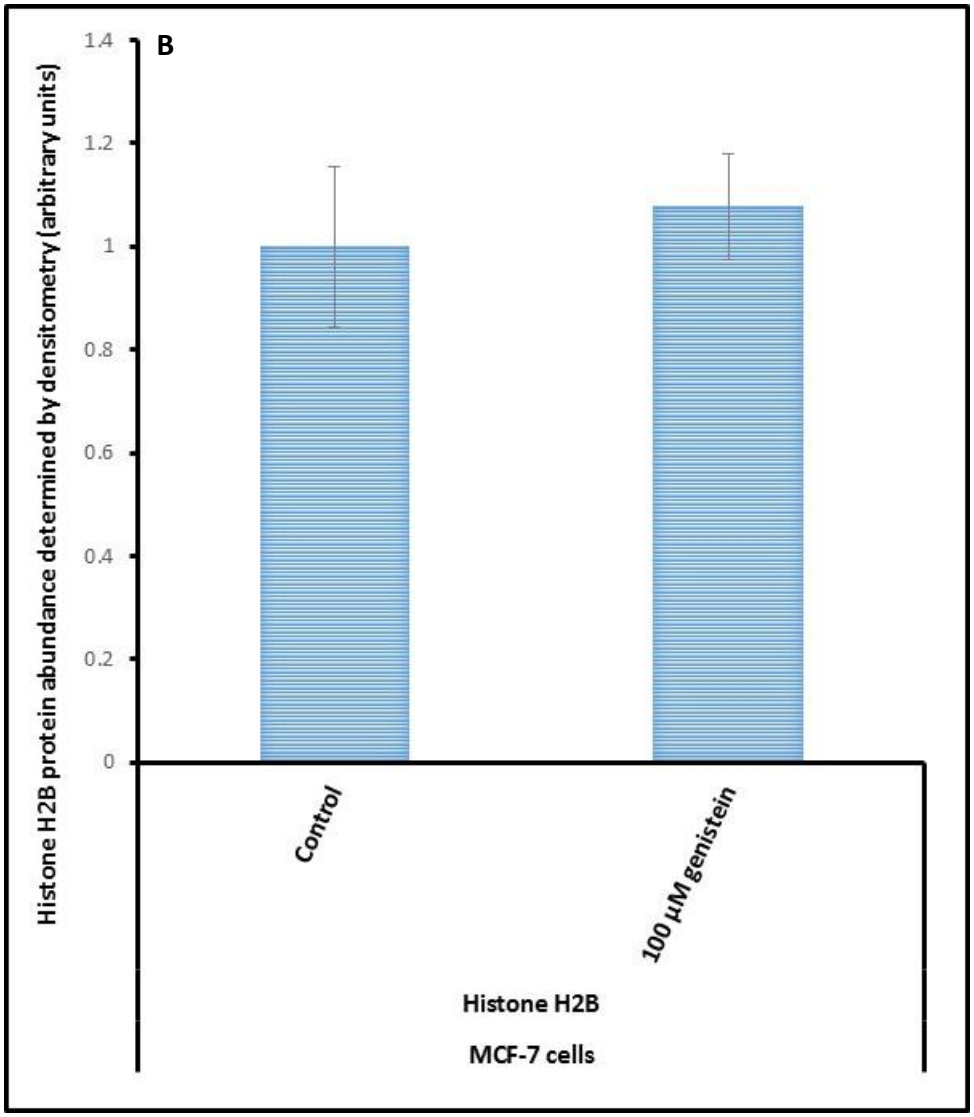


Figure 4. 6: The effect of genistein on histone H2B protein abundance in MCF-7 cells. Forty eight hours after treatment total protein was extracted and histone H2B protein abundance was measured by western blotting. Panel A shows a typical representative western blot. Bands were quantified using Odyssey densitometry software to generate the data shown in panel B. Data are expressed relative to β-actin and normalised to control values. Values are means (n=6; three separate wells for each of two independent experiments), with standard deviations are represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. *P≥0.05.

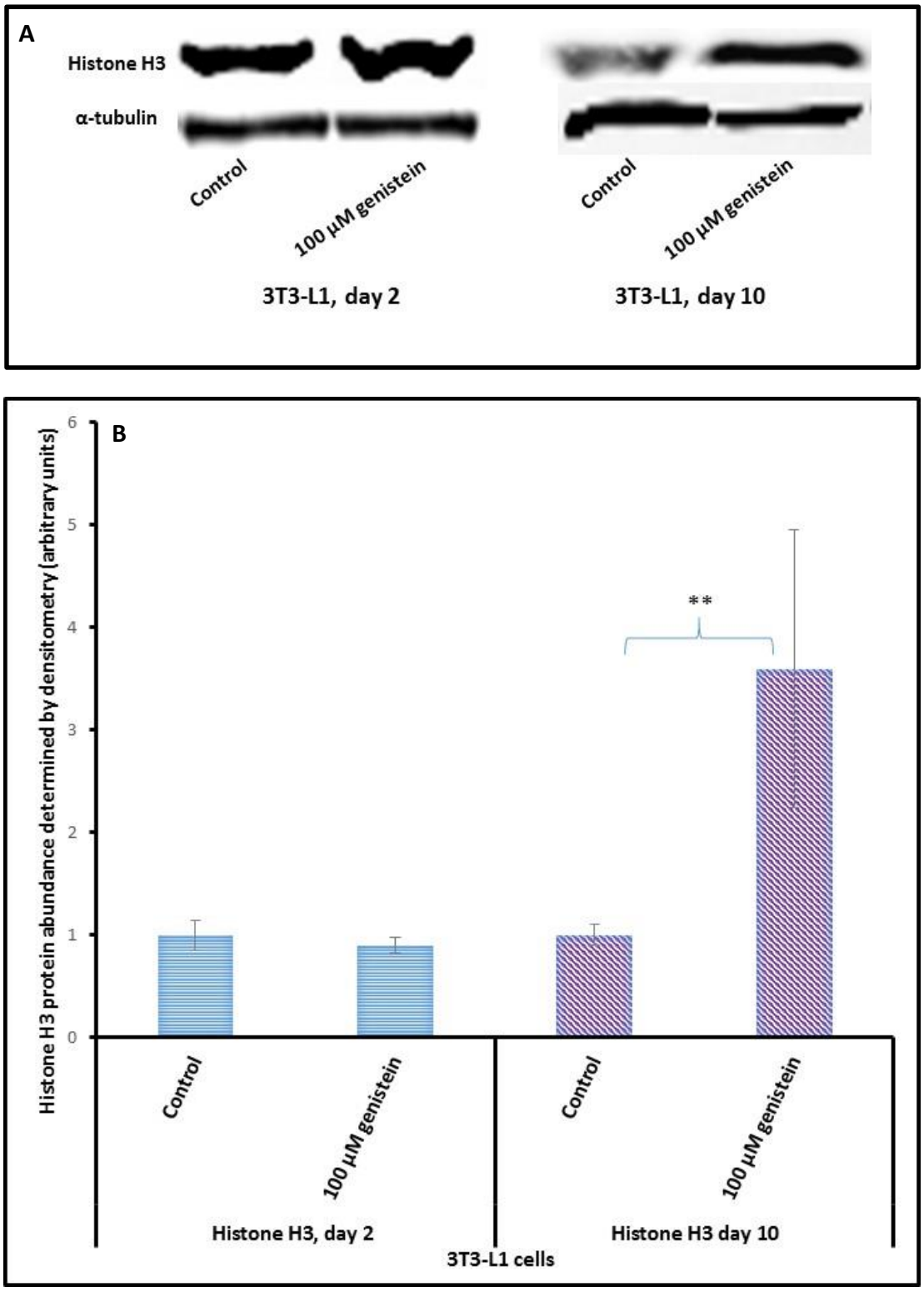


Figure 4. 7: the effect of genistein on histone H3 protein abundance applied on induction of differentiation of 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein. On day 2 and day 10 total protein was extracted. Histone H3 protein abundance was measured by western blotting. Panel A shows a typical representative western blot. Bands were quantified using Odyssey densitometry software to generate the data shown in panel B. Data were expressed relative to α-tubulin and normalised to control values. Values are means (n=5; three separate wells for each of two independent experiments), with standard deviations are represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. **P≤0.01.

4.3.3. The effect of genistein on histone mRNA levels

I reasoned that a possible explanation for the discordant effects of genistein on histone H3 protein and activity of the histone H3.1 (variant H3b) promoter was differential action on different histone variants. H3 variants differ by only a few amino acids and commercially available antibodies such as the antibody used in this work do not differentiate between these variants and thus detect the whole pool of histone H3 variants. I thus used RT-qPCR (primer sequences are stated in chapter 2, tables 1 and 2) to measure the effect of genistein on specific H3 variants. The effect of genistein on 10 individual histone H3.1 variants was measured in MCF-7 and Caco-2 cells using variant specific primers. Concurring with the action of genistein on the H3.1 (Variant H3b) promoter-reporter plasmid construct, most of the histone H3.1 variants measured were down-regulated by genistein (Figures 4.8 and 4.9). However, in agreement with our hypothesis, histone H3.1 variants differed in their response to genistein. In particular in contrast to the other variants histone H3d was upregulated in both cell lines.

I also measured the effect of genistein on histone H3.2 and H3.3 mRNAs in MCF-7 cells and observed a reduction in histone H3.2 mRNA and an increase in histone H3.3 mRNA (Figure 4.10), further showing that genistein has histone H3 variant-specific actions.

The Effect of genistein on the pool of histone H3 variants was also investigated in 3T3-L1 adipocytes using primer that annealed to a region of conserved sequence. In overall agreement with the effect I observed on histone H3 protein, genistein (100μM) applied on induction of differentiation, induced an increase in this H3 mRNA pool, measured at day 12 of differentiation (Figure 4.11).

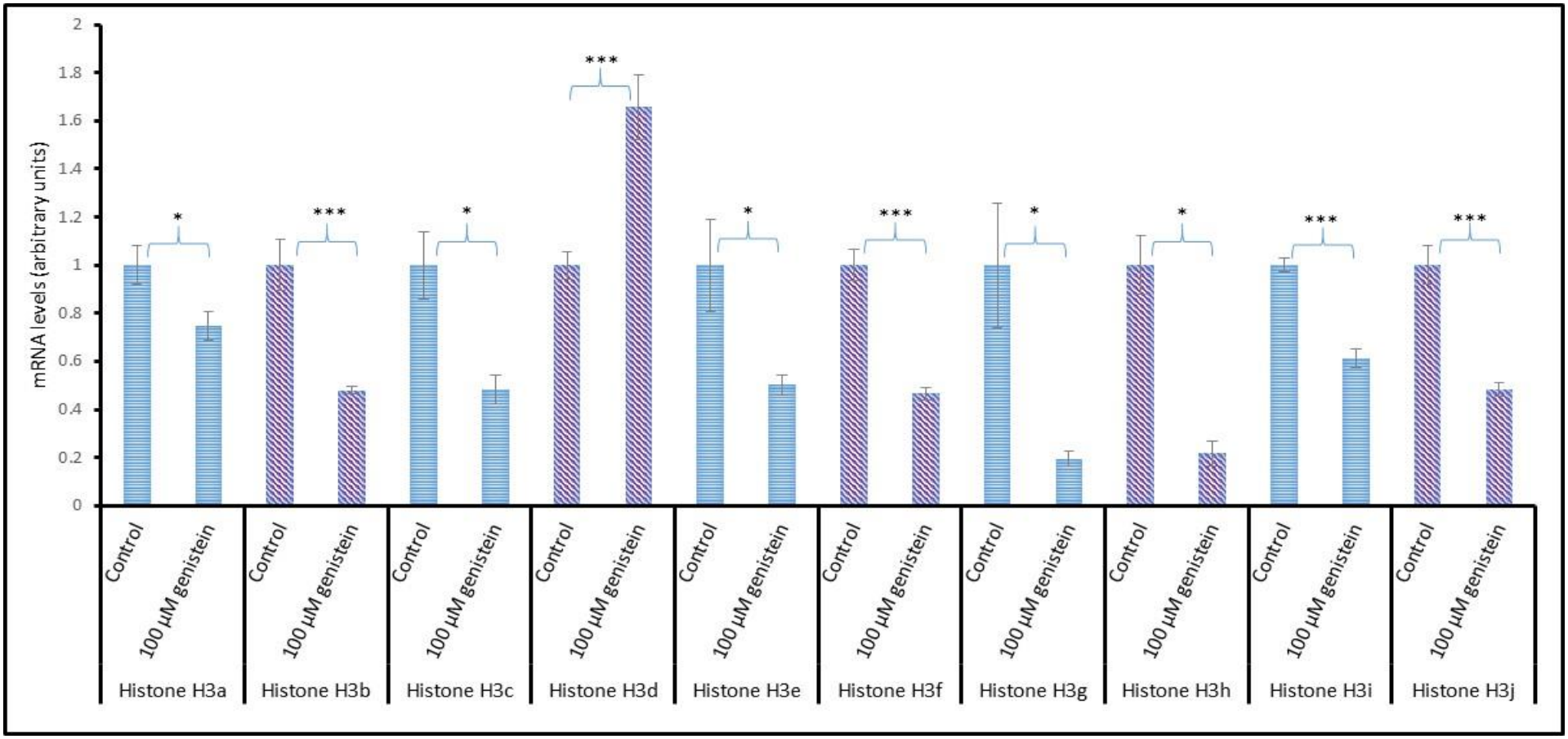


Figure 4. 8: The effect of genistein applied for 48 h on histone H3.1 mRNA levels in Caco-2 cells. Cells were cultured in 6-well plates. Twenty four hours after seeding, cells were treated with genistein (100 µM) for 48 h. Total RNA was extracted and mRNA levels of histone H3.1 variants were measured by RT-qPCR. Data were expressed relative to the reference genes TOPO-1 and GAPDH and normalised to control values. Values are means (n=3; three separate wells for one experiment), with standard errors are represented by vertical bars. Statistical analysis was by Student`s T-test. *P≤0.05, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

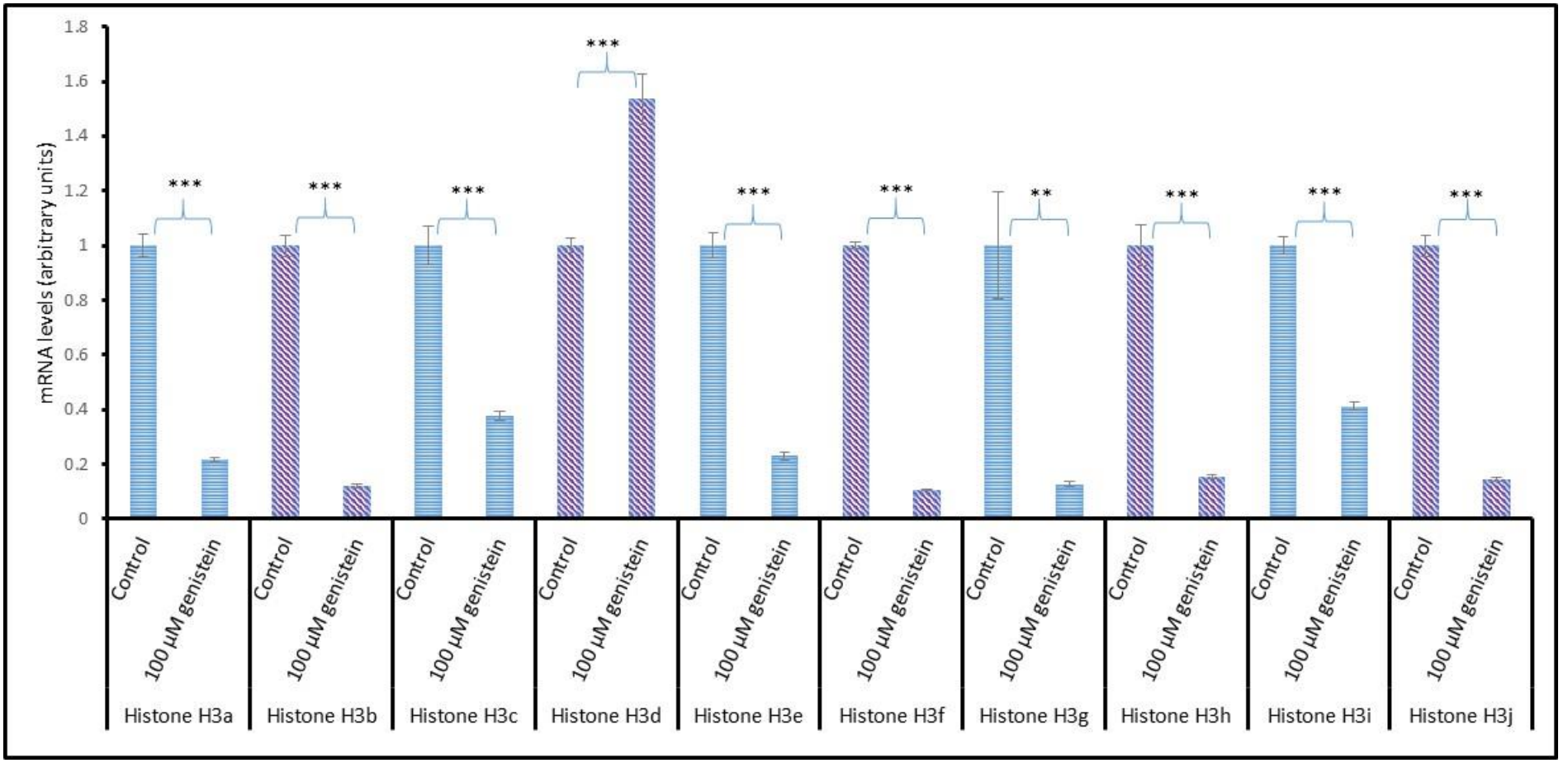


Figure 4. 9: The effect of genistein applied for 48 h on histone H3.1 mRNA levels in MCF-7 cells. Cells were cultured in 6-well plates. Twenty four hours after seeding, cells were treated with genistein for 48 h. Total RNA was extracted and mRNA levels of histone H3.1 variants were measured by RT-qPCR. Data were expressed relative to the reference genes TOPO-1 and GAPDH and normalised to control values. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors are represented by vertical bars. Statistical analysis was by Student`s T-test. *P≤0.05, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

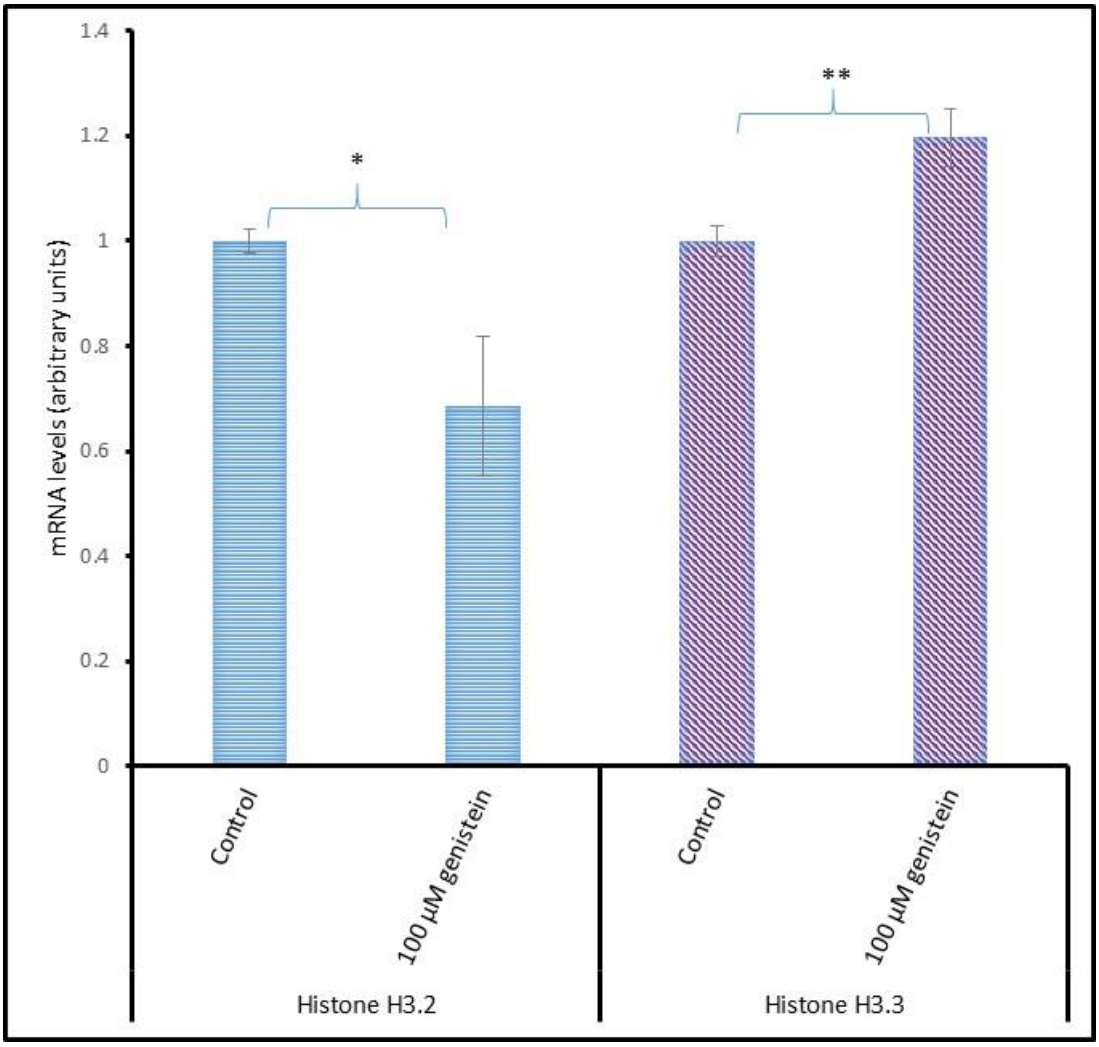


Figure 4. 10: The effect of genistein applied for 48 h on histone H3.2 and H3.3 mRNA levels in MCF-7 cells. Cells were cultured in 6-well plates. Twenty four hours after seeding, cells were treated with genistein (100 µM) for 48 h. Total RNA was extracted and mRNA levels of histones H3.2 and H3.3 were measured by RT-qPCR. Data are expressed relative to the reference genes TOPO-1 and GAPDH and normalised to control values. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors are represented by vertical bars. Statistical analysis was by Student`s T-test. *P≤0.05, **P≤0.01. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

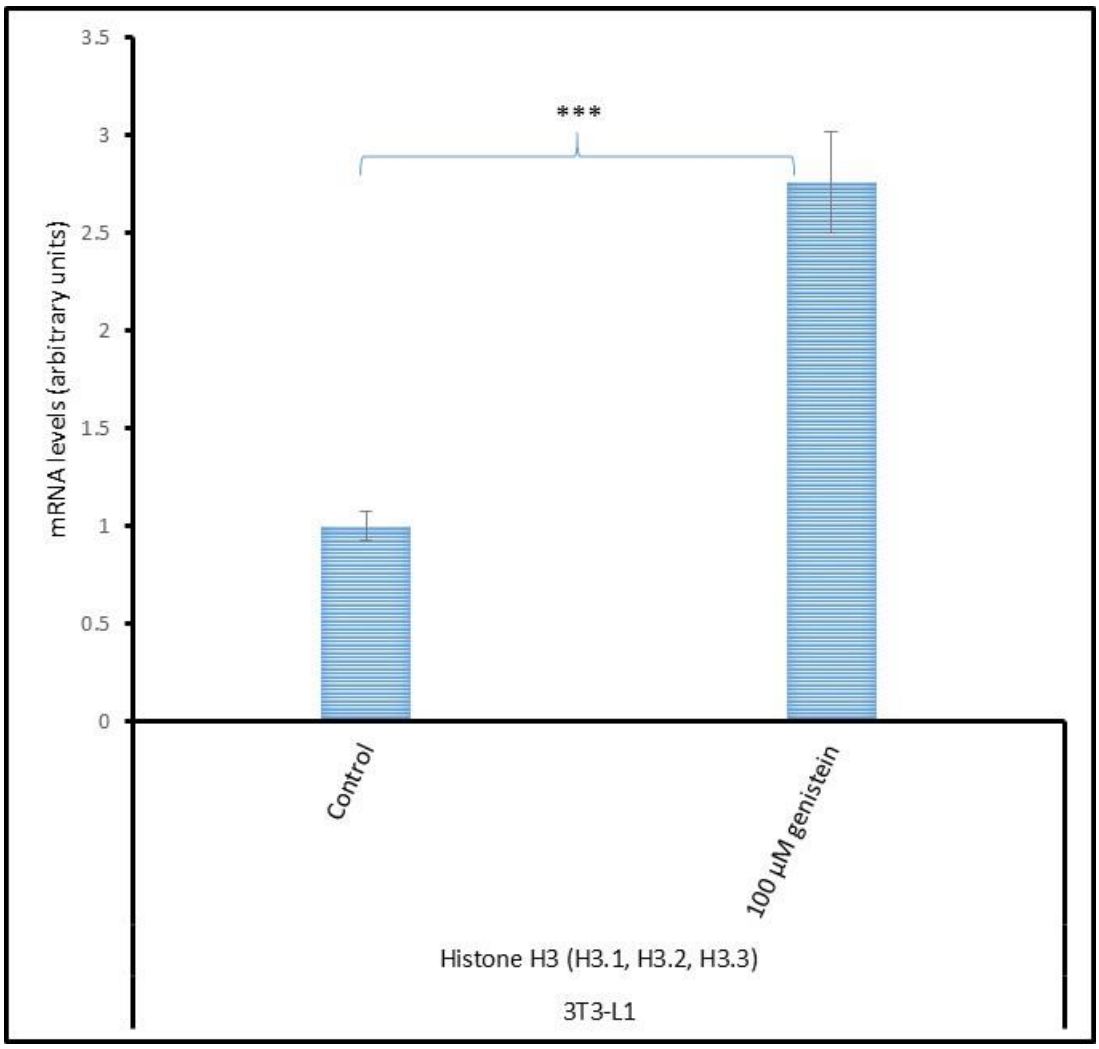


Figure 4. 11: The effect of genistein on histone H3 (H3.1, H3.2 and H3.3) mRNA level applied on induction of differentiation of 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0 and were induced to differentiate in the presence and absence of genistein (100 µM). Total RNA was extracted at day 12 and the mRNA levels of histone H3 were measured by RT-qPCR. Data were expressed relative to the reference genes TOPO-1 and NONO and normalised to control values. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors are represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

4.3.4. The effect of an ER antagonist on the response of histone mRNA levels to genistein

Having found no evidence that genistein action on the histone H3.1 (Variant H3b) promoter was through the ER, since the ER-antagonist fulvestrant did not influence this response, I determined if other elements of action that affected the net abundance of histone H3.1 (variant H3b) mRNA (which could be effects on mRNA stability, translation or protein degradation) were via the ER. MCF-7 cells were treated in the presence and absence of 100 µM genistein and the estrogen receptor antagonist fulvestrant (0.1 µM) or G-15 (1 µM). G-15 (1 µM) is a specific GPR30 antagonist (Dennis *et al.*, 2009; Prossnitz and Barton, 2011) and thus inhibits the GPR30 mediated estrogen response pathway (Hutchens *et al.*, 2012; Luo *et al.*, 2014). Concurring with the data obtained using the histone H3 promoter reporter plasmid construct, the down regulatory effect of genistein on histone H3.1 (variant H3b) mRNA was not attenuated by either of the estrogen receptor antagonists. However, both fulvestrant and G-15 reduced the mRNA levels of histone H3.1 (variant H3b) (Figures 4.12 and 4.13), suggesting that endogenous levels of antagonist compounds usually maintained baseline level.

Similarly in 3T3-L1 cells the response of histone H3 (H3.1, H3.2 and H3.3 combined) mRNA levels to genistein was not reversed by fulvestrant (Figure 4.14).

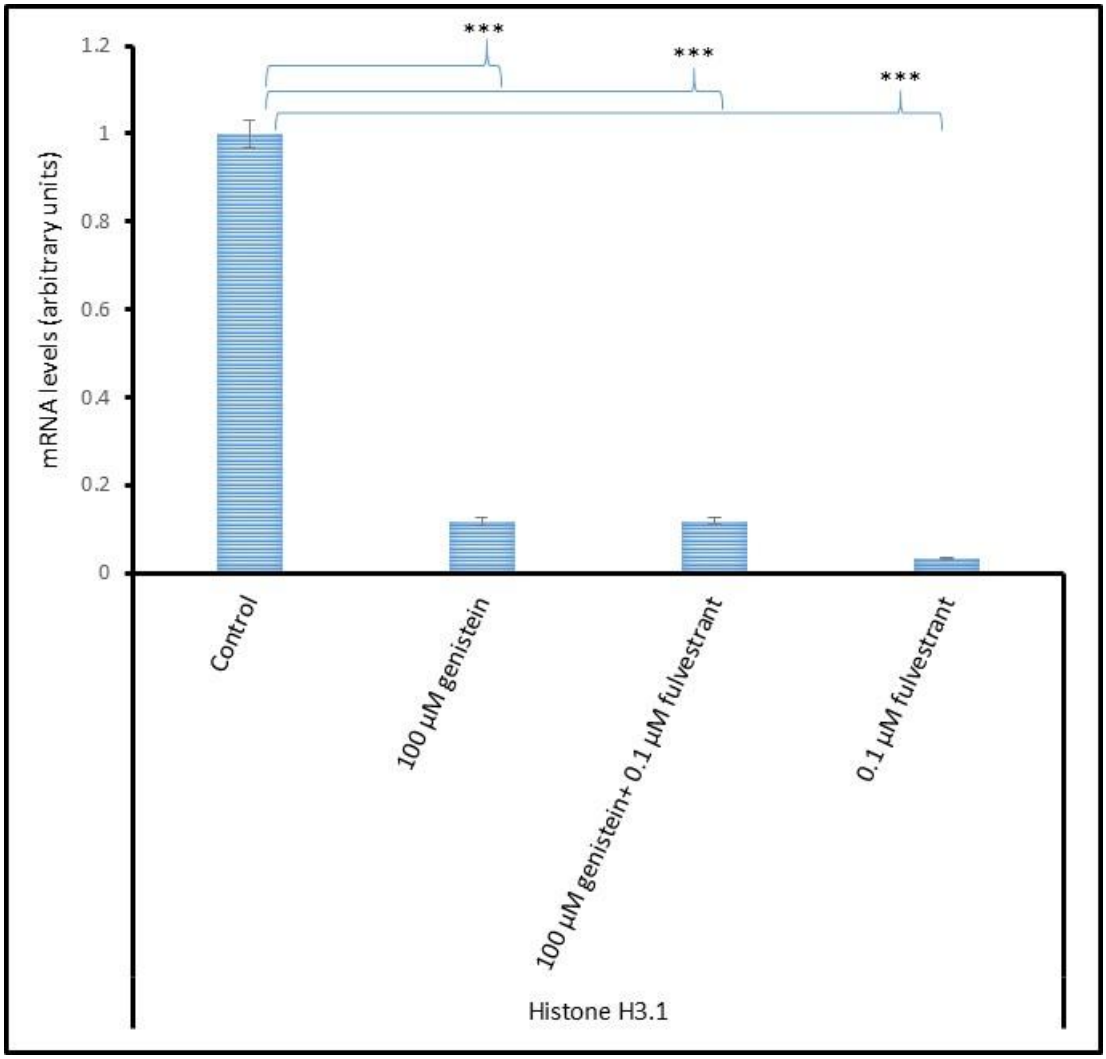


Figure 4. 12: The effect of genistein applied for 48 h on histone H3.1 (variant H3b) mRNA in the presence and absence of the ER antagonist fulvestrant in MCF7 cells. Cells were seeded in 6-well plates. Twenty four hours after seeding, cells were treated with genistein (100 µM) with or without fulvestrant (0.1 µM) for 48 h. Total RNA was extracted and mRNA levels of histone H3.1 (variant H3b) was measured by RT-qPCR. Data are expressed relative to the reference genes TOPO-1 and GAPDH and normalised to control values. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors are represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

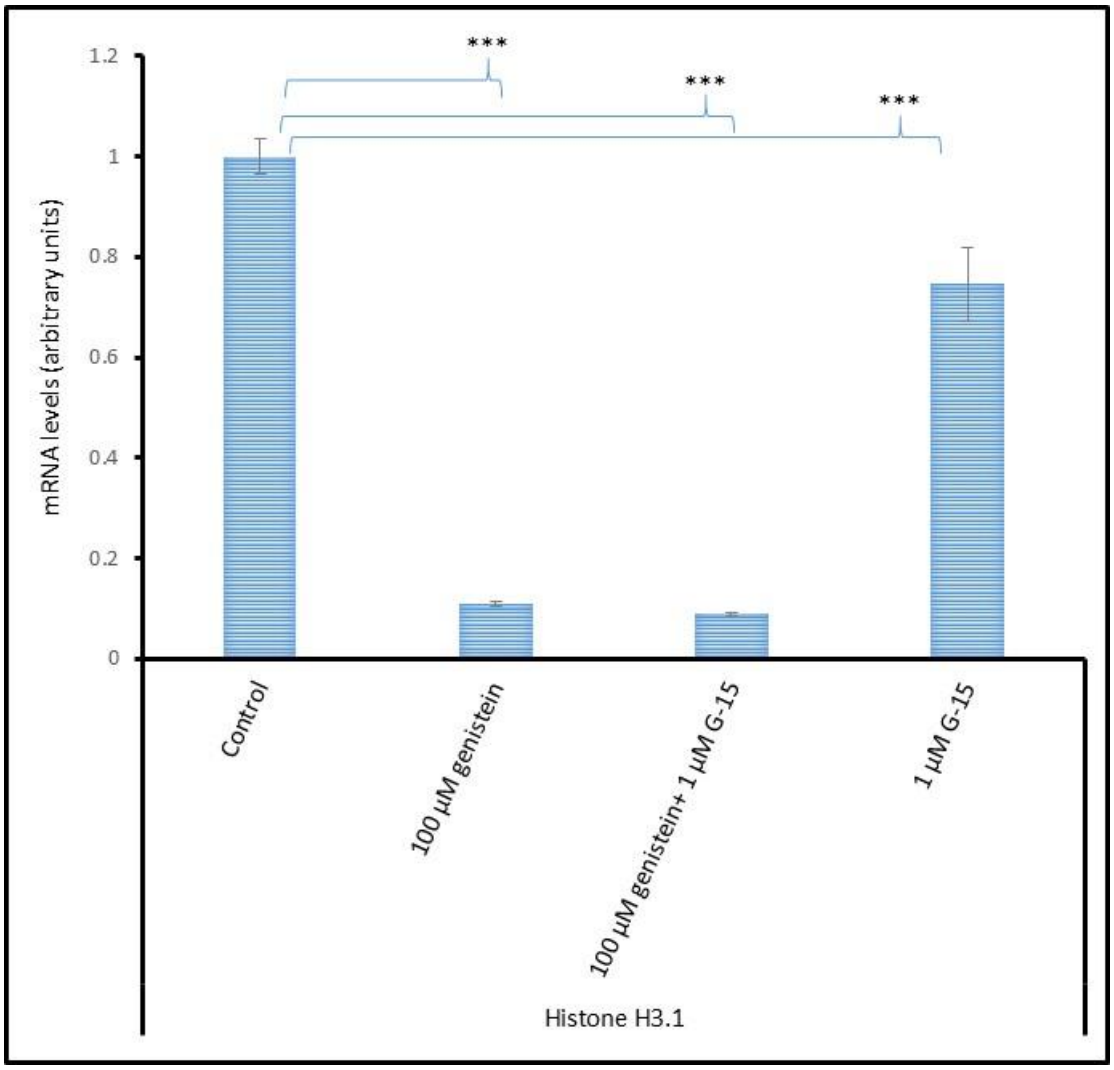


Figure 4. 13: The effect of genistein applied for 48 h on histone H3.1 (variant H3b) mRNA in the presence and absence of the ER antagonist G-15 in MCF7 cells. Cells were seeded in 6-well plates. Twenty four hours after seeding, cells were treated with genistein (100 µM) with or without G-15 (1 µM) for 48 h. Total RNA was extracted and mRNA levels of histone H3.1 (variant H3b) was measured by RT-qPCR. Data are expressed relative to the reference genes TOPO-1 and GAPDH and normalised to control values. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors are represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. . Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant

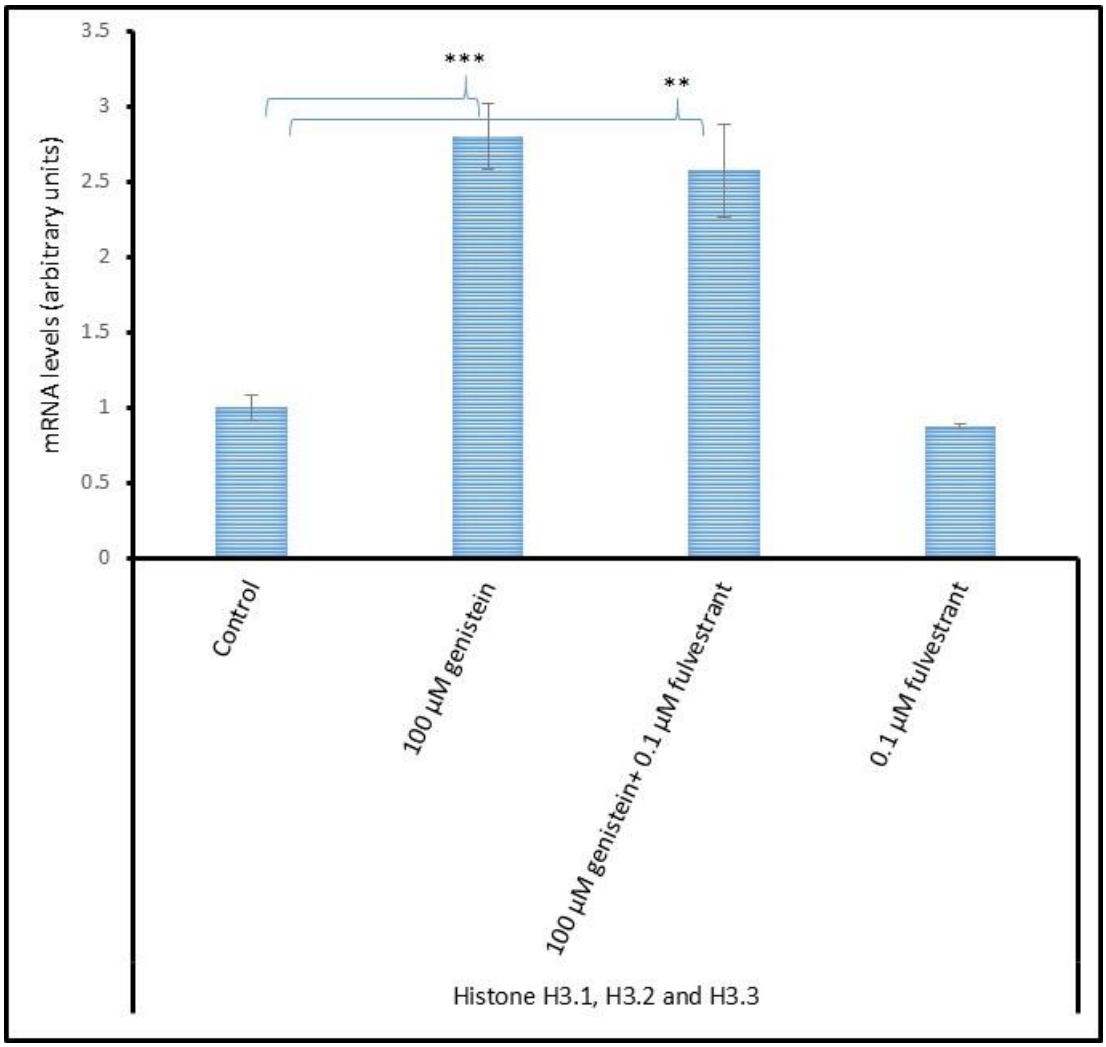


Figure 4. 14: The effect of genistein on histone H3 (H3.1, H3.2 and H3.3) mRNA expression level in the presence and absence of estrogen receptor antagonist (fulvestrant) in 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Fourty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of 100 µM genistein and 0.1 µM fulvestrant. Total RNA was extracted on day 12 and mRNA levels of histone H3 were measured by RT-qPCR. Data are expressed relative to the reference genes Top-1 and Nono and normalised to control values. Values are means (n=3; three separate wells for one experiment), with standard errors are represented by vertical bars, Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. **P≤0.01, ***P≤0.001. . Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant

4.4. Discussion

The results presented in this chapter reveal the effect of genistein and resveratrol alone and in combination on histone expression. Previous work in the laboratory (Alatawi, 2012) revealed that resveratrol reduced the level of histone protein abundance and also the activity of a histone H3.1 (variant H3b) promoter-reporter plasmid in Caco-2 human intestinal cells. I proposed that genistein may produce similar effects because genistein and resveratrol are both polyphenols and have both been shown to have actions mediated via the ER. Secondly, in the natural diet, resveratrol and genistein can occur together, hence their interacting effects are worthy of investigation. I determined if adding both compounds together to the extracellular medium of cultured cells had an effect on histone protein expression or the activity of the histone H3.1 (variant H3b) promoter-reporter construct that was greater than the effect of the single compounds. Caco-2, MCF-7 and 3T3-L1 cell lines were used based on their use to acquire the previous data (Caco-2 and MCF-7 cell) and to investigate effects also in an adipocyte model (3T3-L1), since effects of genistein on other aspects of adipose tissue physiology was the major focus of the work in this thesis. Data acquired during the present study showed that resveratrol and genistein alone and in combination decreased histone H3.1 (variant H3b) promoter activity in Caco-2 cells. These finding confirmed the effect of resveratrol observed previously and were a novel observation in the case of genistien alone and in combination with resveratrol.

The effect of resveratrol and genistein in combination was not greater than that observed with either compound separately, indicating that the compounds probably affect histone H3 gene transcription through the same mechanism. Since both resveratrol and genistein have structural similarity to 17β-estradiol and have been shown to induce effects in some instances through the ER I determined if the action of either of both compound on the histone H3 promoter-reporter construct was via ERs. I thus determined if the ER-antagonist fulvestrant affected the response of the histone H3.1 (variant H3b) promoter-reporter construct to resveratrol and genistein. I found that the response to either resveratrol or genistein was not attenuated in the presence of fulvestrant. Thus, the likely common pathway through which both compounds repress the H3.1 (variant H3b) promoter is unlikely to be through the ER. Other effects of resveratrol reported in

the literature are ER-independent. For example, low (around 0.1 nM) and high (20- 170 µM) concentrations of resveratrol inhibited cell proliferation in both the ER positive cell line MCF-7 and the ER negative cell line MDA-MB-231 (Mgbonyebi *et al.*, 1998; Damianaki *et al.*, 2000). Also the effect of resveratrol on growth inhibition was not reversed by the ER antagonist ICI 182,780 (Levenson *et al.*, 2003). However, other actions of resveratrol appears to be via the ER. For example, resveratrol (0.1 µM) stimulated an increase in glucose uptake by C2C12 cells and the response was abrogated in the presence of the ER antagonist ICI 182,780 (Deng *et al.*, 2008).

Since I found no evidence for synergistic interaction of resveratrol and genistein with regard to action on the histone H3.1 (variant H3b) promoter, and because actions of genistein are the primary focus of the work presented in this thesis, effects of only genistein on histone expression were investigated further. This work comprised measurement of the effect of genistein on histone protein and mRNA in MCF-7, Caco-2 and 3T3-L1 cells to confirm that the response of histone H3.1 (variant H3b) to genistein is ER-independent, the effect of genistein on histone H3.1 (variant H3b) mRNA was measured in the presence and absence of the ER antagonist fulvestrant and also G-15, which is GPR30 antagonist. Consistent with the data on histone H3.1 promoter activity, the response was not attenuated by fulvestrant, Similarly, G-15 had no effect on the action of genistein. These finding further indicate that the effect of genistein on histone protein expression is ER-independent. A limitation of this work is that I did not identify a robust positive control response to show that fulvestrant and G-15 were active antagonists in our experiment models. However, the fact that I observed a significant reduction in the mRNA of histone H3.1 (variant H3b) in response to fulvestrant and G-15 alone (section, 4.3.4) indicated the compounds were active. Also the concentrations and exposure times used have been shown to be effective in published studies (Howell, 2006; Hutcheson *et al.*, 2011; Hutchens *et al.*, 2012; Luo *et al.*, 2014).

Our finding that genistein action was not through the ER is discordant with other reported findings (eg. Zhu et al, (2009). Such differences may relate to the concentrations of genistein used. Studies showing effects of genistein through the ER in different cell models including MCF-7, HeLa, Caco-2, KS483 and mouse bone marrow cells have typically used lower concentrations of genistein(≤3.7 µM) (Maggiolini et al., 2001; Dang et al.,

2003; Chen and Donovan, 2004). The action of genistein at higher concentrations appears to be via other cellular pathways (Dang et al., 2003). Actions measured at higher concentrations include inhibition of tyrosine kinase and topoisomerase activities (Salti et al., 2000; Chen and Donovan, 2004; Kasai and Kikuchi, 2010).

Surprisingly, genistein was found to increase histone H3 protein abundance in Caco-2 and MCF-7, which opposed the effect on histone H3 promoter activity. Similarly, genistein increased histone H3 protein abundance in 3T3-L1 cells. With respect to effects of genistein on other histone proteins I observed that histone H2B protein abundance did not respond to genistein treatment while like histone H3, histone H4 protein abundance was increased. An increase in histone protein abundance appears to have a physiological importance. For instance, reduced global histone protein expression and/or loss of histone proteins at specific regions of the genome is correlated with genome instability and aging. A sharp decrease in the level of histone expression has been observed in aged yeast and aged human foetal lung fibroblast cells (IMR90) (Dang *et al.*, 2009; O'Sullivan *et al.*, 2010; Hu *et al.*, 2014) consistent with an association between higher levels of histone expression and longevity in yeast (Feser *et al.*, 2010a). The expression of histone H3 and H4 particularly was lower in fibroblasts of old versus young human individuals, and at late passage (P75) compared with early passage (P30) of fibroblasts (O'Sullivan *et al.*, 2010). The ratio of histone variants including H2A and H3 variants was also shown to be reduced compared with total histone expression level during aging in human lung embryonic fibroblast cells (Rogakou and Sekeri–Pataryas, 1999). In contrast to a decrease in histone protein abundance with age, histone protein level was found to be increased in aged versus young mice, while there was not any difference in histone protein level between aged and young human intestinal biopsy (Alatawi, 2012).

Histone H3 exists as multiple variants. The antibody used to measure histone H3 did not distinguish between these variants and would thus give only a global readout of net abundance. Histone H3 variants are transcribe from different promoter. Thus I hypothesised that the discordance between the effect of genistein on histone H3 promoter activity and protein abundance may be due to differential effects on specific H3 variants. To test our hypothesis mRNAs for the histone H3 variants including H3.1, H3.2 and H3.3 were measured. In support of our hypothesis genistein was found to decrease

histone H3.2 and H3.1 subvariants with the exception of histone H3d, which was increased. Histone H3.3 mRNAs was also increased.

The significance of this finding with respect to cell physiology is not yet clear. However, specific and distinct roles of histone H3 variants have been shown; thus the findings are likely to be important. For example, the presence of histone H3.3, especially at specific gene promoter and enhancer regions, was associated with transcriptional activity (Ahmad and Henikoff, 2002; Hake and Allis, 2006; Chen *et al.*, 2013a). Expression of this variant was found to be crucial for DNA replication during the S-phase of the cell division (Frey et al., 2014) and to be associated with the transcriptionally active sites of the genome in the chicken erythroid cell models HD24 and 6C2 (Jin and Felsenfeld, 2006). Abundance of this variant marks highly active and fully differentiated cells such as nerve cells (Das and Tyler, 2013). Histone H3.3 knockdown in fertilized mouse zygote resulted in abnormal chromosomal segregation (Bush *et al.*, 2013) and with decondensation of the pericentromeric chromatin (Lin *et al.*, 2013).

Histone H3.2, on the other hand, appears to be associated preferentially with silenced loci and with heterochromatin formation, whereas histone H3.1 seems to have bidirectional (activation and/or silencing) effects on transcriptional regulation (Hake and Allis, 2006). Replacement of histone H3.3 with histone H3.1 prevented C2C12 differentiation into myocytes and prevented lineage specific gene expression (Harada et al., 2014). Also histones H3.1 and H3.2 were found to be correlated with heterochromatin formation during fertilization and embryogenesis in the mouse (Akiyama et al., 2011). Moreover, specific histone variants appear to be susceptible to specific forms of modification. For example histone H3.3 but not H3.1 was found to undergo extensive trimethylation at lysine 4 and 27, which is a hallmark of active chromatin, and to be less susceptible to lysine 9 and 27, dimethylation which are associated with heterochromatin formation (McKittrick et al., 2004; Harada et al., 2014).

The present study is the first, to the best of our knowledge, to demonstrate selective regulatory and ER-independent effects of genistein on histone expression in different cell models comprising Caco-2, MCF-7 and 3T3-L1 cells. Thus, dietary intake of genistein may affect gene expression through specific alterations in chromatin structure

and this may be one of the mechanisms through which this compound has its reported beneficial effects.

Chapter 5

5. Effects of genistein and 17β-estradiol on ER expression

5.1. Introduction

Estrogen receptors (ER) belong to the steroid/thyroid superfamily of intracellular receptors (Evans, 1988). They show wide distribution throughout the body tissues and are involved in physiological and pathophysiological process including growth, development, adipogenesis and cancer (Dunnwald *et al.*, 2007; Lee *et al.*, 2012a). The importance of ER expression in cancer has driven research in this direction. Thus, knowledge about the influence of ER action in this area is extensive. For example, based on epidemiological studies, ERβ was identified as an important marker to classify severity and stage of colon cancer (Rudolph et al., 2012). A substantial reduction and/or absence of ERβ is characteristic of some forms of cancer including colon adinocarcinoma (Foley et al., 2000; Campbell-Thompson et al., 2001; Konstantinopoulos et al., 2003), breast cancer (Skliris et al., 2003; Huang et al., 2014) and prostate cancer (Leav et al., 2001). Overexpression of ERβ can inhibit proliferation of both ERα-positive and ERβ-negative human ovarian cancer cell lines (BG1 and PEO14) (Bossard et al., 2012). Human breast cancers expressing high levels of ERβ have been shown to respond better to hormonal therapy than ERβ negative breast cancers (Murphy et al., 2002). Furthermore, ERβ was found to counteract the proliferative effects of ER-α in the breast cancer cell model T47D and in rat prostate cancer (Ström et al., 2004; Attia and Ederveen, 2012). However, the ERβ knockout mouse showed abnormal growth of breast and prostate epithelial tissue cells, which is a predisposing factor in neoplasia formation (Gustafsson and Warner, 2000; Weihua et al., 2001). In contrast to ERβ, ERα appears to be highly expressed by cancer cells and approximately 70% of breast cancer cases are diagnosed as ER-α positive (DeSantis et al., 2011). Unlike to ERβ, ERα seems to play a role in the onset (Hewitt et al., 2002) and progression (McPherson et al., 2008; Attia and Ederveen, 2012) of cancer.

In contrast knowledge about the role of ER function in adipogenesis, which is of particular relevance to the work in this thesis, is relatively poor. However, there is evidence that both ERα and ERβ may have functions that protect against obesity. An ERβ knockout mouse model fed a high fat diet accumulated more lipid in gonadal adipose tissue compared with wild type (Foryst-Ludwig *et al.*, 2008). In contrast, work in the

ovariectomized mouse indicated that ERβ has no role in diet-induced obesity (Roesch, 2006). However, an ERα was found to be important in reducing food intake and preventing diet-induced obesity in ovacriectomized rats (Roesch, 2006). An ERα knockdown mouse model was obese and developed obesity-related disorders including insulin resistance, glucose intolerance and lower energy expenditure compared with wild type (Heine *et al.*, 2000). Another study showed a reduction in the expression of ERα in obese female subjects compared with lean subjects (Nilsson *et al.*, 2007).

As a ligand, genistein has a higher affinity toward ERβ than ERα (Zhu et al., 2006; Chrzan and Bradford, 2007). Previous published data regarding the effect of genistein on expression of ERs appears to be conflicting. For example giving genistein to mice (50-200 mg/kg) downregulated both ERs in epididymal adipocytes (Penza et al., 2006). A low dose of genistein (1 μM) was found to have no effect on ERβ expression in MCF-7 cells but upregulated ERβ in T47D breast cancer cells. In a study in 3T3-L1 and MCF-7 cells genistein (50-200 μM) was shown to down-regulate ERα dose and time dependently (Choi et al., 2014).

5.2. Objectives

Published work shows that genistein can mediate some of its effects through the ER in different cell models including rat primary neuronal cell culture (Adams *et al.*, 2012), primary mouse bone marrow cells, murine preosteoblastic (KS483) cells (Dang *et al.*, 2003), Caco-2 cells (human intestinal adenocarcinoma cells) (Chen and Donovan, 2004) and MCF-7 cells (breast cancer cell model) (Maggiolini *et al.*, 2001). However, in the present study, as shown by data presented in chapters 3 and 4, the effects of genistein on adipocyte phenotype and histone expression that I observed appeared to be ER-independent. However, it was still possible that genistein influenced ER expression in our cell models under the experimental conditions applied. This view was based on the fact that genistein and 17β-estradiol are structurally similar and can have similar actions and because 17β-estradiol (1-20 nM) has been shown to down-regulate expression of ERα in different breast cancer cell lines including M6 cells (Yoshidome et al., 2000), MCF-7 cells (Hall et al., 2008) and ZR-75-1 cells (Kousidou et al., 2008). In contrast, ERβ expression was not affected by 17β-estradiol in SW480 intestinal cancer cells (Hartman et al., 2009). Our aim thus was to test the hypothesis that genistein, like 17β-estradiol, can differentially

regulate ERα and ERβ. The objective was to measure the effect of 100 μM genistein on the expression of both ER subtypes, and compare this action with that of 17β-estradiol. There is evidence that expression of the ER can be auto-regulatory (Cardone *et al.*, 1998). The second objective was therefore to determine if any effects of genistein on ER expression I observed were themselves mediated via the ER.

The approach was to treat MCF-7 and 3T3-L1 cells with genistein and 17β-estradiol including under conditions of ER blockade by antagonists fulvestrant and G-15, and then measure mRNAs of ERα and ERβ by RT-qPCR. Primer sequences are stated in chapter 2, tables 1 and 2**.**

5.3. Results

5.3.1. The effect of genistein or 17β-estradiol on ERα and ERβ expression

Initially the effect of 17β-estradiol on ER mRNA levels was measured by RT-qPCR in MCF-7 cells, which express both ER subtypes. Twenty four hours after seeding, cells were treated with 2 nM 17β-estradiol for 48 h. I observed that 17β-estradiol decreased ERα mRNA but did not have any effect on ERβ mRNA (Figure 5.1).

To determine if genistein has any effect on ER subtype expression, MCF-7 cells were treated with 100 μM genistein for the same length of time (48 h). The data revealed that the two ER subtypes were differentially regulated by 100 μM genistein. Similar to the observations we made using 17β-estradiol, genistein reduced ERα mRNA. However, unlike 17β-estradiol, which had no effect, genistein increased ERβ mRNA (Figure 5.2).

To investigate if these effects of genistein on ER subtypes were cell type specific or if they are observed in other cell types, the effect of genistein on ER mRNA was measured in 3T3-L1 adipocytes at day 12 after induction of differentiation in the presence and absence of 100 μM genistein. The effects of genistein on ER mRNAs in 3T3-L1 cells were consistent with those observed in MCF-7 cells; ERα mRNA was decreased and ERβ mRNA was increased (Figure 5.3).

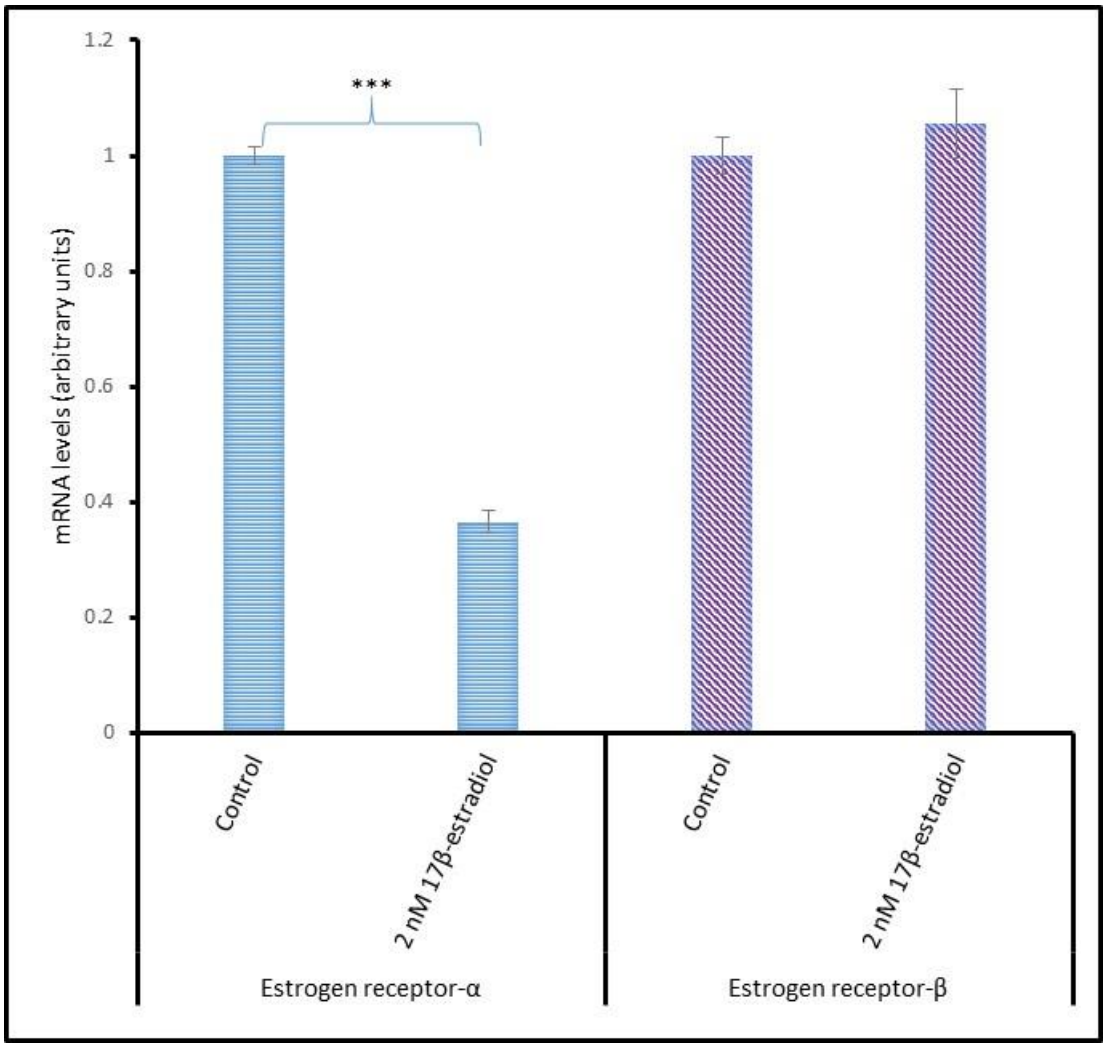


Figure 5. 1: The effect of 17β-estradiol on the ER-α and ER-β mRNAs in MCF-7 cells. Cells were grown in 6-well plates. Twenty four hours after seeding cells were treated with 17β-estradiol (2 nM) for 48 h, total RNA was extracted and mRNA levels of ER-α and ER-β were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and GAPDH and normalised to control. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant

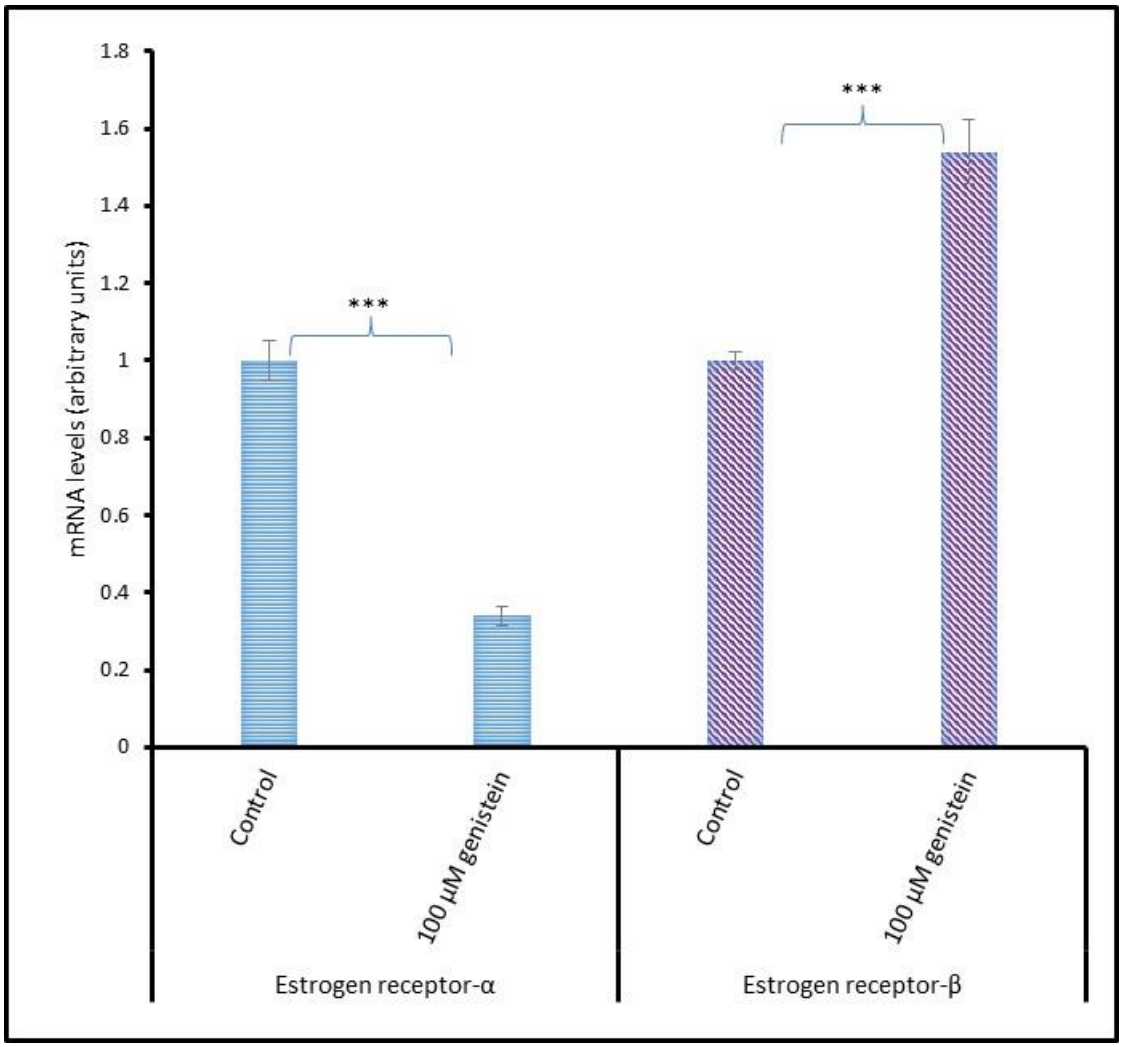


Figure 5. 2: The effect of genistein on ERα and ERβ mRNAs in MCF-7 cells. Cells were grown in 6-well plates. Twenty four hours after seeding cells were treated with genistein (100 µM) for 48 h. Total RNA was extracted and mRNA expression levels of ERα and ERβ were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and GAPDH and normalised to the control. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant

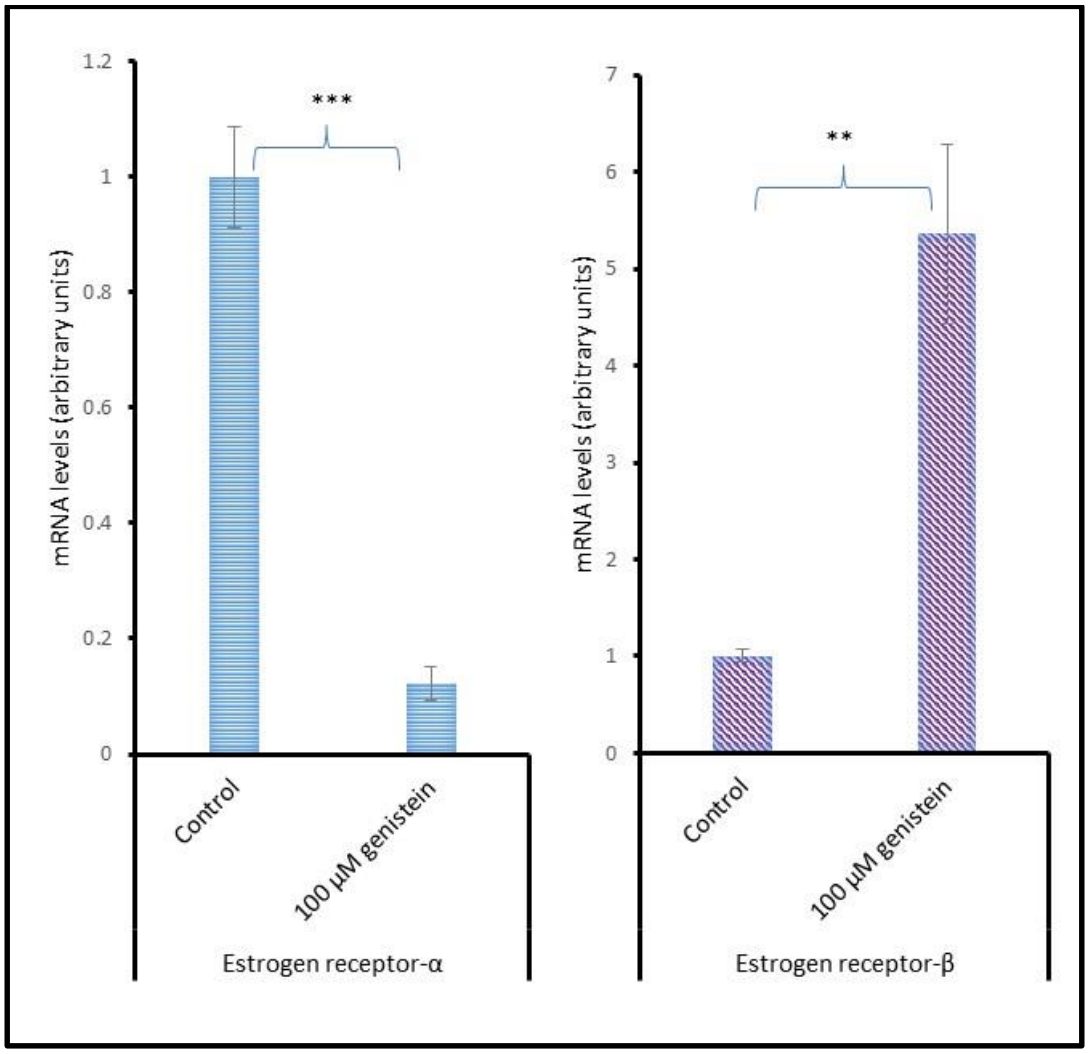


Figure 5. 3: The effect of genistein on the ERα and ERβ mRNAs applied on induction of differentiation in 3T3-L1 cells. Cells were grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of genistein (100 µM). Total RNA was extracted at day 12. mRNA levels were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and NONO and normalised to control. Values are means (n=3; three separate wells for one experiment), with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. **P≤0.01, ***P≤0.01. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

5.3.2. The effect of ER antagonists on the response of ERα and ERβ mRNAs to genistein or 17β-estradiol

To investigate if the effect on ER mRNA was auto-regulatory (mediated through the ER), MCF-7 cells were treated for 48 h with genistein in the presence and absence of the ER-antagonists fulvestrant or G-15 (Figure 5.4). Fulvestrant (0.1 μM) is a potent ER antagonist that blocks both the AF1 and AF2 domains of the ER with no agonist effect. Upon binding it prevents ER dimerization and translocation into the nucleus and also promotes receptor degradation (Howell, 2006; Hutcheson *et al.*, 2011). G-15 (1 μM) (Hutchens *et al.*, 2012; Luo *et al.*, 2014) is a specific antagonist of the non-classical membrane G protein coupled ER GPR30 (Dennis *et al.*, 2009; Prossnitz and Barton, 2011; Chakrabarti and Davidge, 2012; Chevalier *et al.*, 2012).

Measurement of ERα and ERβ mRNAs by RT-qPCR showed that neither ER antagonist affected the response to genistein (Figures 5.5 and 5.6) showing that these actions of genistein are independent of any binding to ERs. However, fulvestrant alone increased ERα mRNA (Figure 5.5), indicating that endogenous levels were repressed by the actions of compounds in the tissure culture medium (possibly 17β-estradiol) whose actions were blocked by fulvestrant. To determine if genistein and 17β-estradiol differed with respect to potential auto-regulatory effects on ER expression, MCF-7 cells were treated with 2 nM 17β-estradiol in the presence and absence of 0.1 μM fulvestrant or 1 μM G-15. In contrast to the action of genistein, the down-regulatory effect of 17β-estradiol on ERα was attenuated in the presence of fulvestrant, indicating an auto-regulatory action of ERα on its own expression (Figure 5.7). As in the earlier experiments we also observed that fulvestrant alone increased ERα mRNA (Figure 5.7). Consistent with the data obtained in the previous experiment (Figure 5.1), ERβ was not affected by 17β-estradiol (Figures 5.7 and 5.8). Together, 17β-estradiol and fulvestrant induced a very small but statistically significant reduction in ERβ mRNA (Figure 5.7).

G-15 had no effect on either the response of ERα mRNA to 17β-estradiol or on the levels of ERα or ERβ mRNA measured in the absence of 17β-estradiol indicating that the GPR30 pathway does not play a role in the action of 17β-estradiol on ER expression (Figure 5.8).

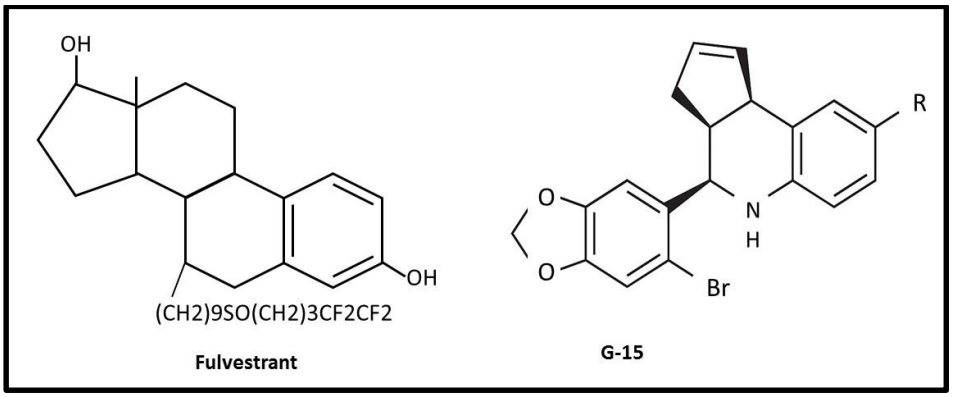


Figure 5. 4: Chemical structure of fulvestrant and G-15

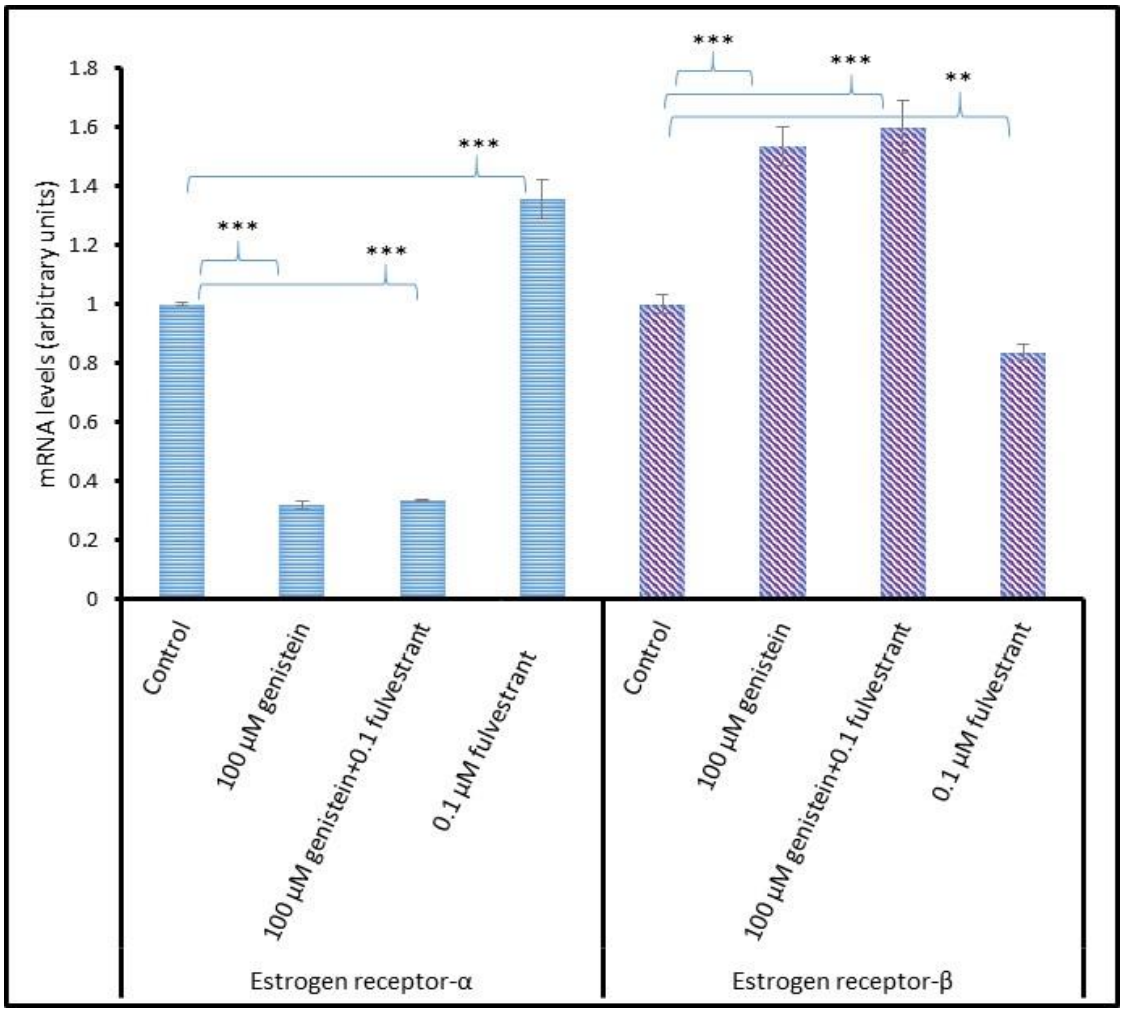


Figure 5. 5: The effect the ER antagonist fulvestrant on the response of ERα and ERβ mRNA to genistein in MCF-7 cells. Cells were grown in 6-well plates. Twenty four hours after seeding cells were treated with genistein (100 µM) in the presence and absence of fulvestrant (0.1 µM) for 48 h. Total RNA was extracted and mRNA levels of ERα and ERβ were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and GAPDH and normalised to control. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

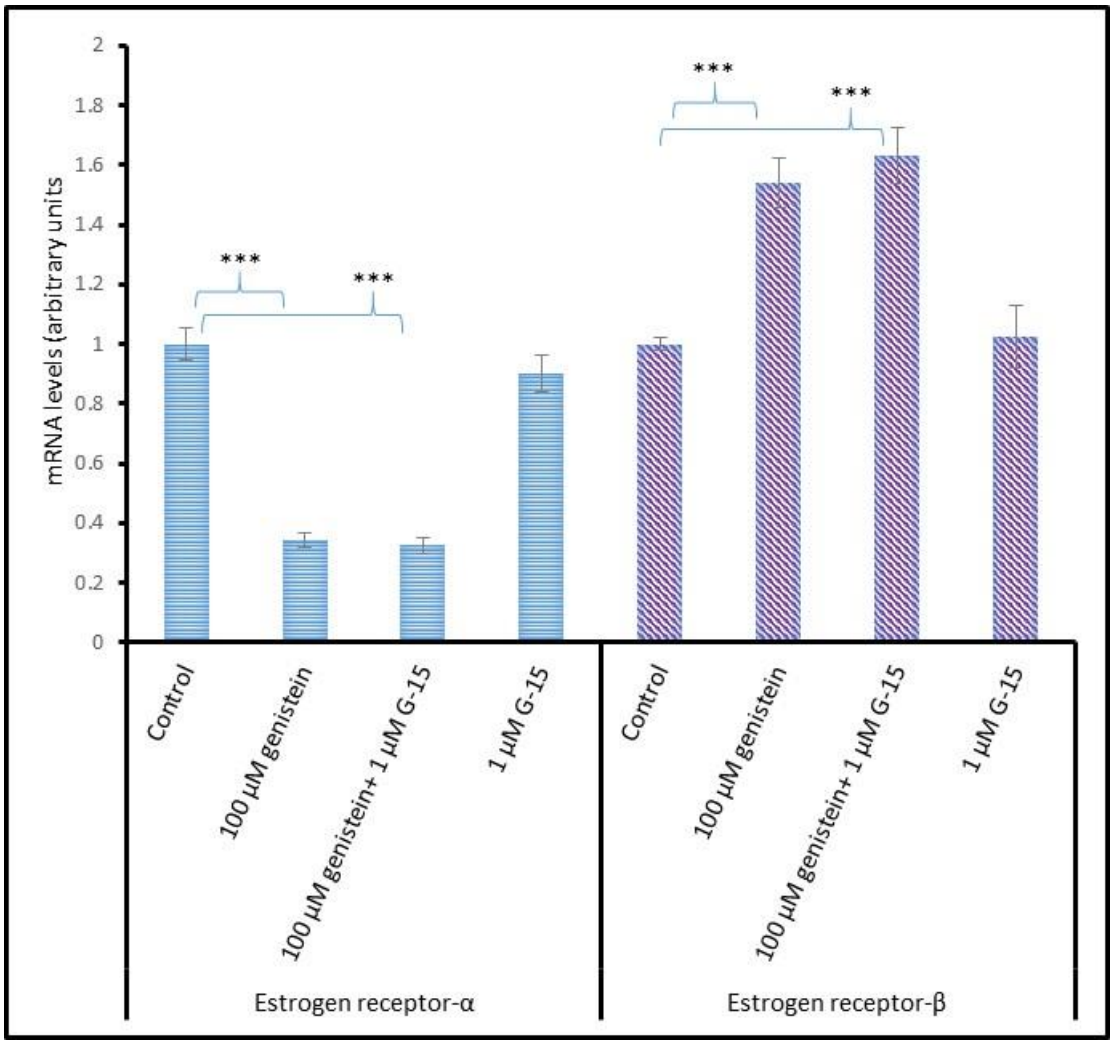


Figure 5. 6: The effect of the ER antagonist G-15 on the response of ERα and ERβ mRNA to genistein in MCF-7 cells. Cells were grown in 6-well plates. Twenty four hours after seeding cells were treated with genistein (100 µM) in the presence and absence of G-15 (1 µM) for 48 h. Total RNA was extracted and mRNA levels of ERα and ERβ were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and GAPDH and normalised to control. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

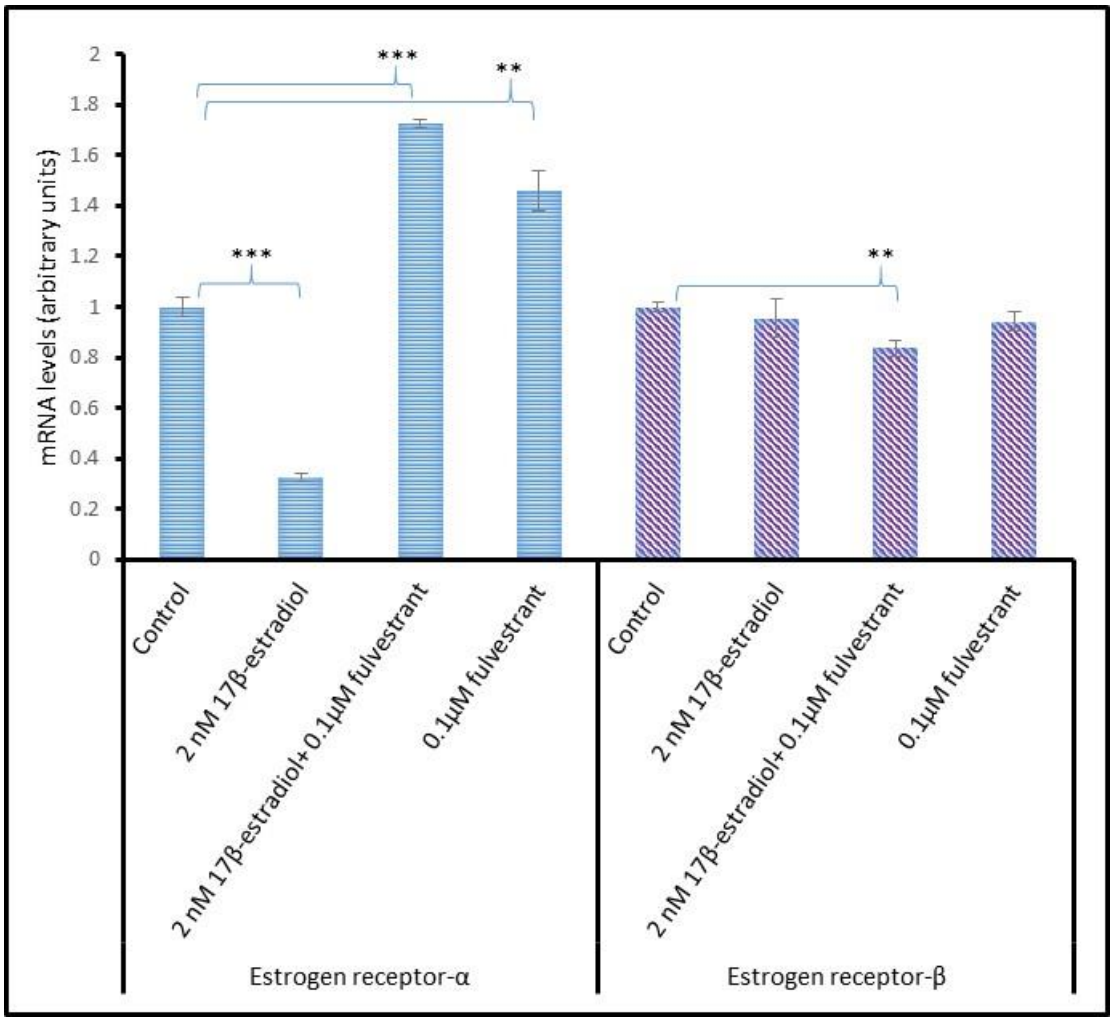


Figure 5. 7: The effect of the ER antagonist fulvestrant on the response of ERα and ERβ mRNA to 17β-estradiol in MCF-7 cells. Cells were grown in 6-well plates. Twenty four hours after seeding cells were treated with 17β-estradiol (2 nM) in the presence and absence of fulvestrant (0.1 μM) for 48 h. Total RNA was extracted and mRNA levels of ERα and ERβ were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and GAPDH and normalised to control. Values are means (n=3; three separate wells for one experiment), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

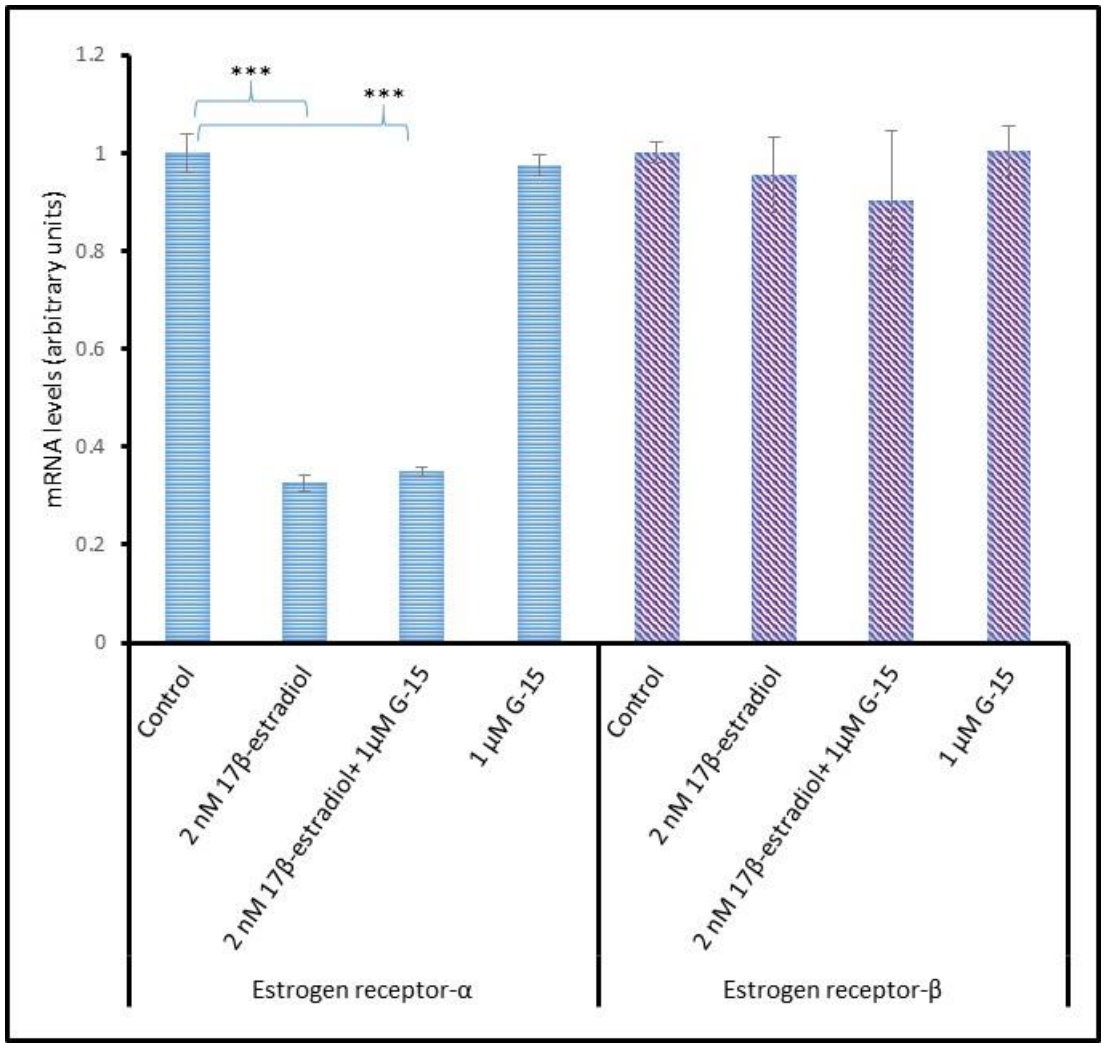


Figure 5. 8: The effect of the ER antagonist G-15 on the response of ERα and ERβ mRNA to 17β-estradiol in MCF-7 cells. Cells were grown in 6-well plates. Twenty four hours after seeding cells were treated with 17β-estradiol (2 nM) in the presence and absence of G-15 (1 μM) for 48 h. Total RNA was extracted and mRNA levels of ERα and ERβ were measured by RT-qPCR. Data are expressed relative to the reference genes Topo1 and GAPDH and normalised to control. Values are means (n=3; three separate wells for one experiment), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

5.3.3. Investigating if genistein increases ERβ mRNA by increasing transcription of ERβ gene

The effect of genistein on ERβ mRNA was of particular interest because of the possible therapeutic benefits in cancer prevention or treatment of increasing ERβ expression (Murphy *et al.*, 2002; Bossard *et al.*, 2012). Thus, I probed the mechanism by addressing the hypothesis that the point of action is transcription of the ERβ gene. Also, despite the fact that I saw no evidence that the effect of genistein on ERβ was itself an ER-mediated response (because ER antagonists had no effect), I tested the effect of ER antagonists because I identified likely EREs in the human ERβ promoter (Figure 5.9). I reasoned that measuring directly the action of genistein and the influence of ER antagonists by use of a promoter-reporter construct may be more sensitive than measurement of ERβ mRNA. I constructed an ERβ promoter-reporter construct comprising 1600 bp of the region of genomic DNA immediately upstream of the transcription start site of the ERβ gene upstream of a β-galactosidase reporter gene (Figure 5.9). The required promoter region was amplified from genomic DNA by PCR using primers (chapter 2, table 3) to add *Hind* III and *EcoR*1 restriction sites 5" and 3" of the sequence respectively. Then both vector (pSF-pA-PromMCS-BetaGal) and the amplified DNA insert were digested with *Hind* III and *EcoR*1, and the DNA insert was subcloned upstream to the β-galactosidase reporter gene as described in chapter 2 section 2.7. After transforming DH5α *E. coli* then purifying the plasmid construct, successful subcloning of the DNA insert was confirmed by digesting the recombinant plasmid construct with *Hind* III and *EcoR*1 and observing the products after agarose gel electrophoresis. The plasmid construct was sequenced to further confirm correct identity of the cloned DNA insert.

Caco-2 cells were transfected transiently with the ERβ promoter reporter plasmid construct. Twenty four hours after transfection, cells were treated with 100 μM genistein then β-galactosidase activity was measured after 24, 48 or 72 h. Genistein did not affect the activity of the reporter gene measured after 24 h but at 48 and 72 h after addition genistein increased expression of the reporter gene as measured by an increase in β-galactosidase activity (Figure 5.10). The data thus showed that genistein increases transcription of the ERβ gene.

Consistent with the lack of any effect on the genistein-induced increase in ERβ mRNA, neither fulvestrant nor G-15 attenuated the stimulatory effect of genistein on the ERβ promoter reporter gene construct, indicating that the effect was not an ER-mediated auto-regulatory action (Figures 5.11 and 5.12).

GTGGTGTATGCCTGTAATCCCAGCTACTCAGGAGGCTGAGACATGATAATTGCTTGAACCTGGGAGGCAGAGATTG
CAGTGAGCTGATATGGCGCCATTGCACTCCAGCCTAGGCAACAAGAGTGAAACTCCATCTCAAAAAAAAAGATTTGC
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GGGAGGCAGGAGGTTGCAGTGAGCCGAGATCATGCCACTGCACTCCAGCCTGGGTGACAGAGTGAGACCTTGTCTC
AAAAACAAAGACAAAACCAAAACAAAACAAAACTGAGAAGCAACAGATTGATAAGTGACACAGTTACACTGGTCAG
TCTCTTCAGCTAATACCCATTGTTTTTTATTATTGGAGATTCATAATGTGTTTTCTTTCTTTTAAAAACTTTTTTCGGAAA
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CAGGCACATGCCACCATTCCTGGCTAATTTTTAGTAGAGACGGGGTTTCACCATGTTGCCCAGGCTGGTCTCGAACTC
CTGACCTCAAGTAATCTGCCCACCTCAGCCTCCCAAAGTATTGGGATTACAGGCGTGAGCCACTACGCTTGGCCTCA
TAGCGTATTTTAATATTGGTTGAGACTAGCCTTGCTCATTGATCTTCTCTTAGCGTTTACTTGGTTATTCTTGCTTATTT
TTCCATAAGAACTTTCATTTTTATTTAATCCTGTGTTTTTTGGTTTTAAAGACTATTTTATAATAAATTTTCGTGATTAAA
CTCTTGTGCTTAAACTCTTGATTAAACAAACAAGCAATGAAGAGATGAATGAAGCAGAAAATGTGAGTTTCATGCCT
CACATTCCCACTCCTCTGAGGTTAATATTTTCATGTATATTTTTCAGGATGTATTTGTAATCTCATACAAACGTATGTAT
TTTTTTAATGAAAATATTTAAATTTTCATAGTTAACAGCTGTAGCTCTAACTTGGCAATATCTTCTGTGTTTCTTTACAG
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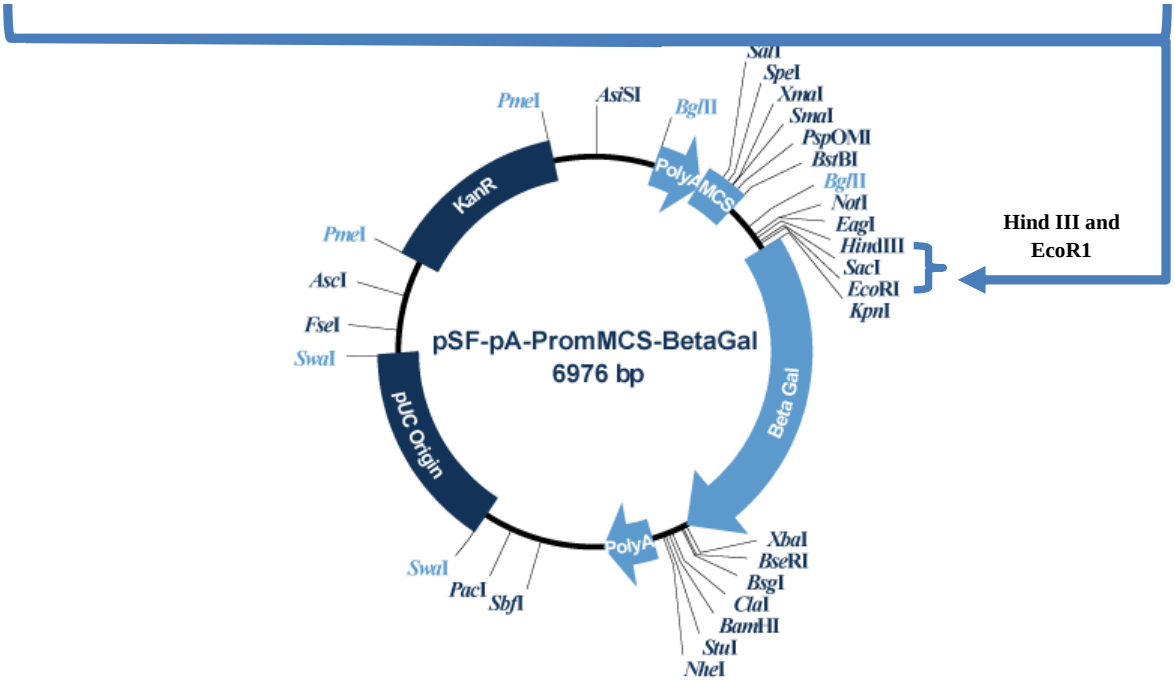

Figure 5. 9: Diagramatic representation of the human ERβ promoter sequence (NC_018925.2) in pSF-pA-PromMCS-BetaGal vector highlighting the occurrence of multiple potential EREs (underlined and in red) in the promoter region. The yellow highlighting shows primers used to amplify the insert from human genomic DNA.

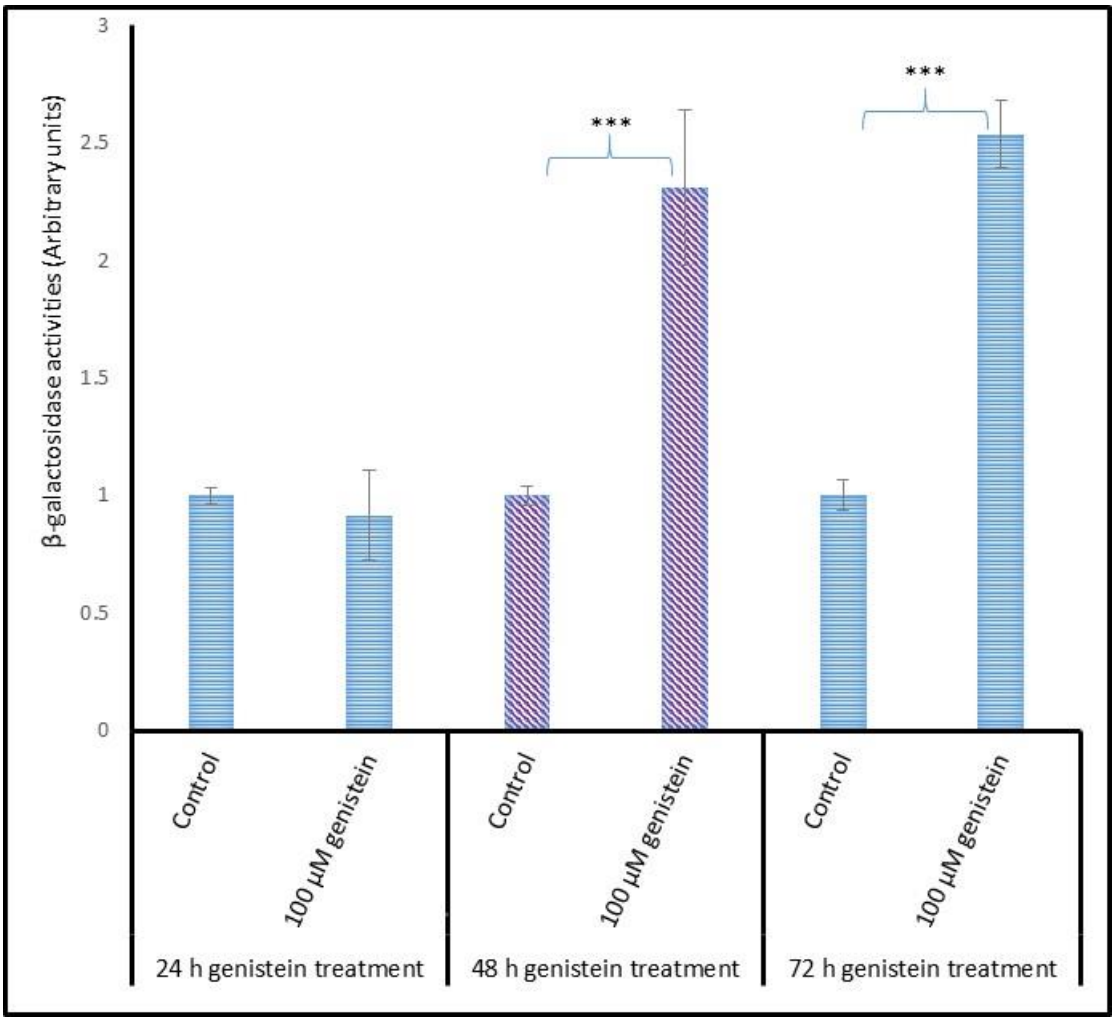

Figure 5. 10: The effect of genistein (100µM) on the response of the human ERβ promoter reporter plasmid. Data are derived from Caco-2 cells transfected with ERβ promoter-reporter plasmid construct comprising 1600 bp upstream to the ER transcription start site gene. Cells were treated with genistein (100 µM) 24 h after transfection and reporter gene activity was measured after further 24 h (n=3; three separate wells for one experiment), 48 h (n=9; three separate wells for each of three independent experiments)) or 72 h (n=9; three separate wells for each of three independent experiments)) of the treatment. Data are expressed relative to total protein and normalised to control. Values are means with standard errors represented by vertical bars. Statistical analysis was by Student's unpaired T-test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

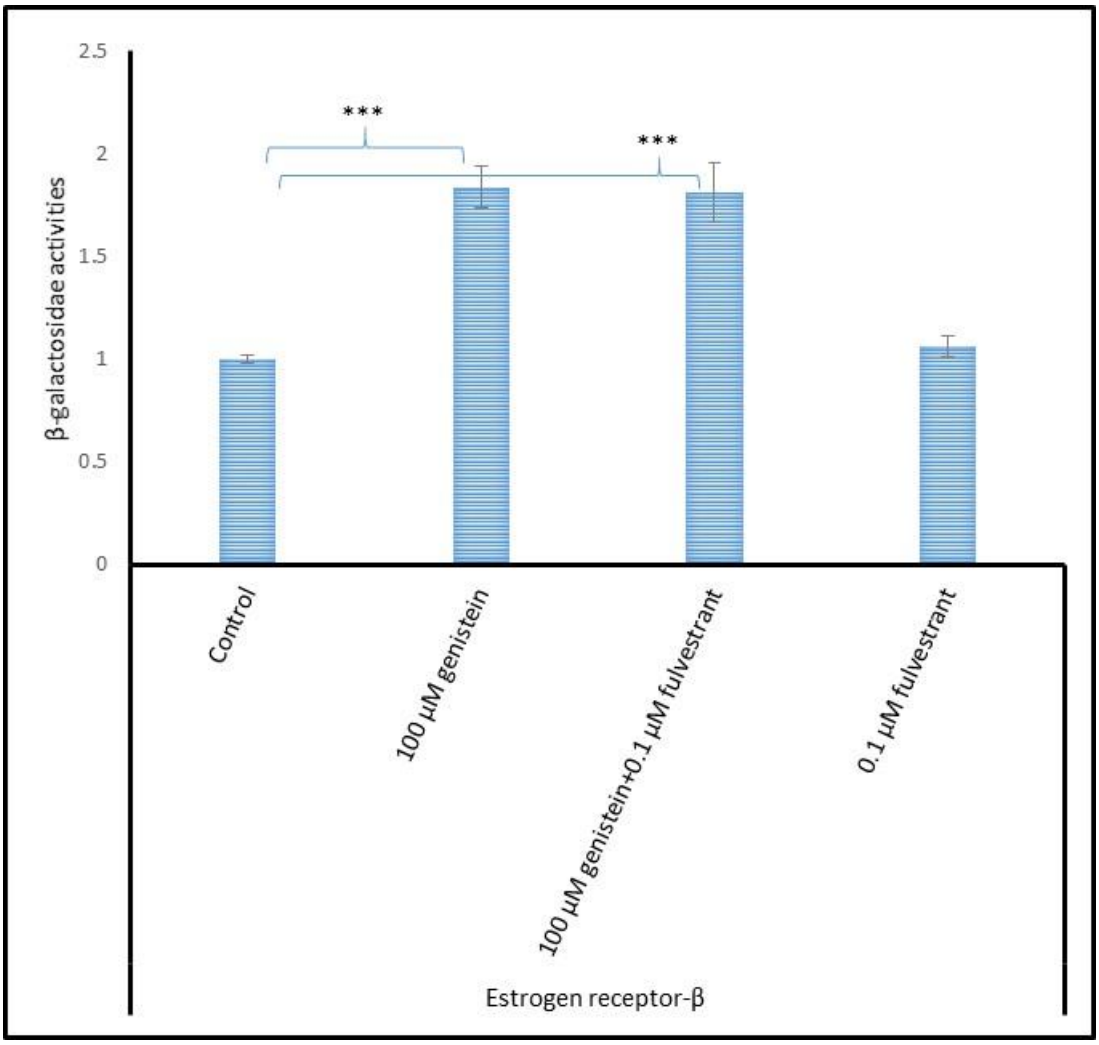

Figure 5. 11: The effect of the ER antagonist fulvestrant on response of the human ERβ promoter reporter plasmid to genistein. Data are derived from Caco-2 cells transfected with ERβ promoter-reporter plasmid construct comprising 1600 bp upstream to the ER transcription start site gene. Cells were treated with genistein (100 µM) in the presence and absence of fulvestrant (0.1 µM) 24 h after transfection and reporter gene activity was measured after further 48 h of the treatment. Data are expressed relative to total protein and normalised to control. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

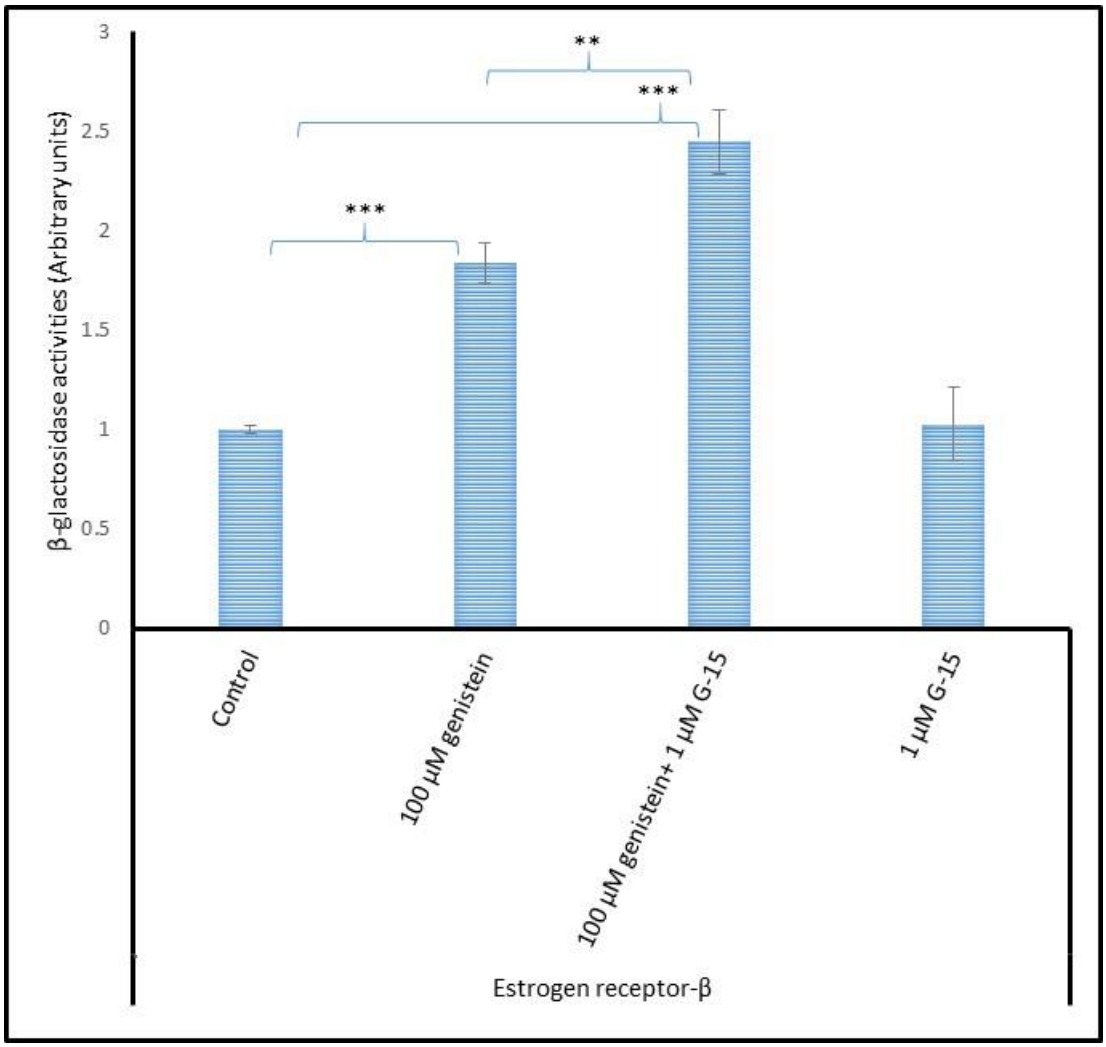

Figure 5. 12: The effect of the ER antagonist G-15 on response of the human ERβ promoter reporter plasmid to genistein. Data are derived from Caco-2 cells transfected with ERβ promoter-reporter plasmid construct comprising 1600 bp upstream to the ER transcription start site gene. Cells were treated with genistein (100 µM) in the presence and absence of G-15 (0.1 µM) 24 h after transfection and reporter gene activity was measured after further 48 h of the treatment. Data are expressed relative to total protein and normalised to control. Values are means (n=6; three separate wells for each of two independent experiments), with standard errors represented by vertical bars. Statistical analysis was by one-way ANOVA followed by Bonferroni's multiple comparisons test. **P≤0.01, ***P≤0.001. Data were distributed evenly around the mean value, as established using Levene`s test for homogeneity of variant.

5.4. Discussion

The aim of the work presented in this chapter was to investigate if there is functional similarity between 17β-estradiol, which is a mammalian endogenous female sex hormone, and genistein with respect to the effects of the two compounds on the expression of ERs. I determined if genistein affects the expression of ERs and if it has any auto-regulatory effect on ER expression such as has been observed for 17β-estradiol (Cardone *et al.*, 1998). Thus, I measured the effect of genistein (100 μM) on the expression of both ER subtypes in MCF-7 cells, and compared this effect with that of 17β-estradiol. The experiment were then repeated under the inhibitory effects of the ER antagonists fulvestrant and G-15.

I found that the two ER subtypes were differentially regulated by genistein. Similar to the action of 17β-estradiol, genistein decreased ERα mRNA. However, in contrast to 17β-estradiol, which had no effect, genistein increased ERβ mRNA. The down-regulation of ERα concomitant with an increase in the expression of ERβ may be of benefit in cancer prevention or treatment. Based on epidemiological (Rudolph et al., 2012) and experimental studies, most cancers, including colon adinocarcinoma (Foley et al., 2000; Campbell-Thompson et al., 2001; Konstantinopoulos et al., 2003), ovarian cancer (Rutherford et al., 2000; Bossard et al., 2012), breast cancer (Skliris et al., 2003; Huang et al., 2014), prostate cancer (Leav et al., 2001) and lung cancer (Kawai et al., 2005), have been associated with a substantial reduction and/or absence of ERβ. Moreover, overexpression of ERβ has been observed to inhibit proliferation of both ERα positive and negative human ovarian cancer cell lines (BG1 and PEO14) (Bossard et al., 2012). Similarly, overexpression of ERβ diminished cell proliferation and enhanced cell cycle arrest at G1 in colon cancer cell models (SW480 and HCT-116) (Hartman et al., 2009). Also human breast cancers expressing high levels of ERβ have been shown to respond better to hormonal therapy than ERβ negative breast cancers (Murphy et al., 2002). Conversely, the ERβ knockout mouse showed abnormal growth of breast and prostate epithelial tissue cells, which is a predisposing factor in neoplasia (Gustafsson and Warner, 2000; Weihua et al., 2001). Again, ERβ knockdown by siRNA in a prostate cancer cell model (LNCaP) increased the proliferative rate of the cells in association with a sharp increase in the expression level of ER-α (Stettner et al., 2007).

In contrast to ERβ, ERα appears to be highly expressed by cancer cells and approximately 70% of breast cancer cases are diagnosed as ER-α positive (DeSantis et al., 2011). Unlike to ERβ, ERα seems to play a role in the onset (Hewitt et al., 2002) and progression of cancer (McPherson et al., 2008; Attia and Ederveen, 2012). Recently it was shown that an ERα dependent action inhibited P53/P21 expression and stimulated expression of proliferative cell nuclear antigen (PCNA) and proliferation-related Ki-67 antigen in MCF-7 breast cancer cells (Liao et al., 2014). A low level of ERα expression was measured in normal human breast tissue but its expression was higher in some forms of breast cancer including invasive ductal carcinoma (Gustafsson and Warner, 2000) and in lobular cancer (Huang et al., 2014). ERα was consistently expressed in patients with non-small cell lung cancer were ERβ expression was variable (Kawai et al., 2005). However, in colon cancer ERα expression was reported to be similar to the surrounding normal tissue, and appeared to have no influence in colon carcinogenesis (Foley et al., 2000; Campbell-Thompson et al., 2001).

Our observations relating to probing the possible auto-regulatory actions of genistein and 17β-estradiol on ER expression also uncovered differences in action between the two compounds, whereas the effect of 17β-estradiol to reduce ERα mRNA was itself an ER-mediated response, being attenuated by the ER-antagonist fulvestrant, the response of neither ERα nor ERβ to genistein was reversed in the presence of fulvestrant, showing that both responses occur through a mechanism that does not involve ERs themselves. The response of the two ERs to neither genistein nor 17β-estradiol was reversed in the presence of the GPR30 antagonist G-15, showing that the response of the ERs to these agents was not modulated through GPR30.

Consistent with the data on effects of genistein and ER antagonists on ERβ mRNA, the ERβ promoter was activated by genistein and this action was not reversed in the presence of either ER-antagonist (fulvestrant and G-15). These observations, similar to those reported in chapters 3 and 4, uncovered effects of genistein that are ER-independent and are consistent with previous studies showing that higher concentrations of genistein such as used in the present work act generally through other mechanisms. These alternative mechanisms of genistein action include inhibition of tyrosine kinase and topoisomerase activities (Salti *et al.*, 2000; Dang *et al.*, 2003; Chen and Donovan, 2004;

Kasai and Kikuchi, 2010). Further work is required to determine if either of these pathways is the mechanism through which genistein has the effects I report here. Lower concentrations of genistein (up to 3.7 µM) appear to mediate biological effects through ERs (Chen and Donovan, 2004; Magee and Rowland, 2004).

In summary I observed that genistein reduced ERα mRNA and increased ERβ mRNA. Neither action appeared to be an auto-regulatory response mediated through ERs. In contrast, 17β-estradiol did not affect ERβ mRNA, while like genistein it reduced ERα mRNA. This was via an ER-mediated auto-regulatory mechanism. The most significant finding reported in this chapter is that genistein can simultaneously reduce ERα and increase ERβ, indicating that the therapeutic potential for prevention or treatment of hormone-dependent cancers may be worthy of future study.

Chapter 6

6. An investigation into DNA methylation as a mechanism through which resveratrol affects d the expression of genes involved in lipid synthesis in 3T3-L1 cells.

6.1 Introduction

DNA methylation status varies depending on cell phenotype and stage of differentiation (Morgan et al., 1999; Chong et al., 2007). The process is controlled by the DNA methyltransferase enzymes (DNMTs) (Okano *et al.*, 1999; Chong *et al.*, 2007). DNA methylation status is very susceptible to modification by environmental and dietary factors including stress and diet (Junien and Nathanielsz, 2007; Daxinger and Whitelaw, 2010; Ford et al., 2011; Uriarte et al., 2013). DNA methylation is considered to be an important likely contributor to the process of aging (Maegawa et al., 2010). Older individuals generally have lower global DNA methylation than younger individuals (Gomes *et al.*, 2012). DNA methylation also appears to be involved in the control of lipogenesis. For example, DNA methylation of the leptin gene varies among different cell types and stages of cell differentiation (Stöger, 2006), being hypermethylated in preadipocytes then hypomethylated in mature adipocytes (Melzner et al., 2002). Furthermore, DNA methylation of the *Fasn* and *Srebp1* promoters genes coding for the enzymes and transcription factor which have important functions in lipogenesis was decreased in mice in response to a high energy diet (Uriarte et al., 2013).

Since there is already such an extensive body of research that shows the activity of genistein on DNA methylation, I selected the second polyphenol included in the work that forms the body of this thesis-resveratrol-for initial work to explore effects on DNA methylation in 3T3-L1 adipocytes. The focus on resveratrol also developed work carried out in the laboratory previously that uncovered effects of resveratrol on the expression of enzymes involved in lipid metabolism.

Published evidence that resveratrol affects DNA methylation includes the observation that when given in the diet to rats it increased in the DNA methylation of genes encoding pro-inflammatory cytokines including IL1β, IL-6, TNF-α and IFN-γ, and reduced their expression (Lou et al., 2014). Resveratrol may alter DNA methylation through affecting DNMT expression and activity (Paluszczak et al., 2010). Resveratrol was

observed to down-regulate expression of DNMT1 (Qin et al., 2005; Stefanska et al., 2012) and DNMT3b (Qin et al., 2005; Qin et al., 2014).

6.2. Objective

Previous observations made in the laboratory by Goiuri Alberdi Aresti were that the mRNA of lipogenic enzymes including fatty acid synthase (FAS) and acetyl Co-A carboxylase (ACC) were reduced in 3T3-L1 adipocytes after treatment with resveratrol. This action may be the basis of a reported action of resveratrol to protect against obesity (Lin *et al.*, 2012b; Jin *et al.*, 2013; Andrade *et al.*, 2014a). We thus proposed that resveratrol may induce changes in the expression of *Acaca* and *Fasn* genes through mediating changes in DNA methylation of *Acaca* and *Fasn* promoters.

The approach was to treat 3T3-L1 cells with resveratrol. Following extraction of DNA and RNA, DNA methylation of *Acaca* and *Fasn* promoters as well as corresponding mRNAs were measured by pyrosequencing and RT-qPCR respectively.

6.3. Results

To exclude a possible effect of cell differentiation on the methylation status of the *Acaca* and *Fasn* promoters prior to investigating the effects of resveratrol, DNA methylation of the promoter regions of these genes was measured at day 0 (before differentiation) and 12 days after initiating the process of differentiation by pyrosequencing. The assay for *Fasn* measured DNA methylation at 5CpG sites at positions -324, -328, -330, -337,-344, -351, -366, -368 relative to the transcription start site. The assay for *Acaca* measured DNA methylation at 5CpG sites at positions -1956, -1972, -1983, -1998, -1916 relative to the transcription start site. Primer sequences are stated in chapter 2, table 4. I found that DNA methylation of neither gene at the CpG sites measured was changed when cells differentiated (Figure 6.1). Consistent with the earlier observations, treatment of cells with 10 µM resveratrol for 48 h (day 10 to day 12 of differentiation) decreased FAS and ACC mRNA (Figure 6.2). However, DNA methylation of the CpGs measured in *Fasn* and *Acaca* genes was not affected by resveratrol applied between day 10 and 12 or applied on induction of differentiation until day 12 (Figures 6.3 and 6.4). The data thus indicate that resveratrol affects the expression of *Fasn* and *Acaca* genes in 3T3-L1 cells by a mechanism other than DNA methylation of the promoters of corresponding

genes, at least at the CpG sites measured in the assay. It is possible that the treatment affected methylation of CpG sites not induced in the assay.

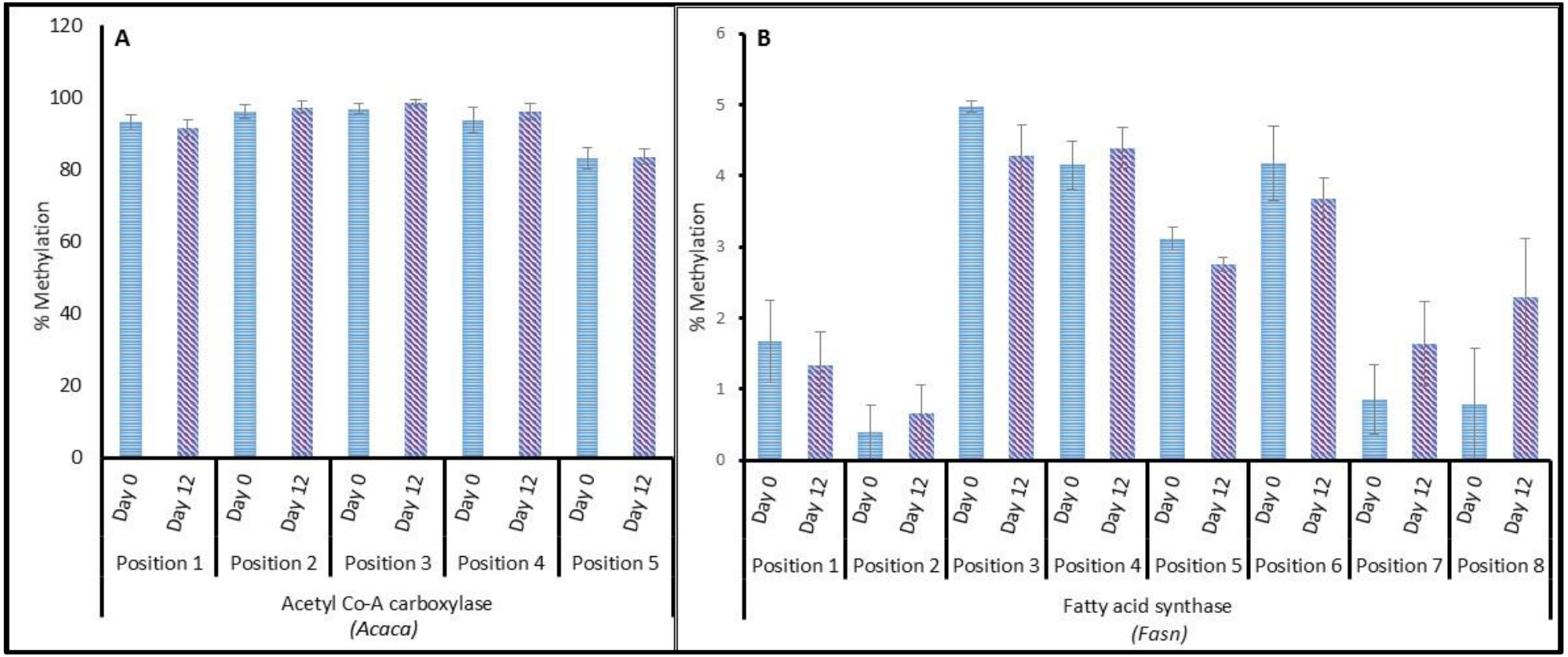

Figure 6. 1: DNA methylation at CpG sites in acetyl co-A carboxylase (ACC; Acaca) (paired A) and fatty acid synthase (FAS; Fasn) (paired B) gene promoter regions in 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate. Genomic DNA was extracted on day 0 and day 12 of differentiation. DNA methylation at specific CpG sites was measured by pyrosequencing. Values are means (n =5; A, n=4; B), with standard errors represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. There were no differences between methylation at day 0 compared with day 12 at any of the CpG sites measured. P> 0.05.

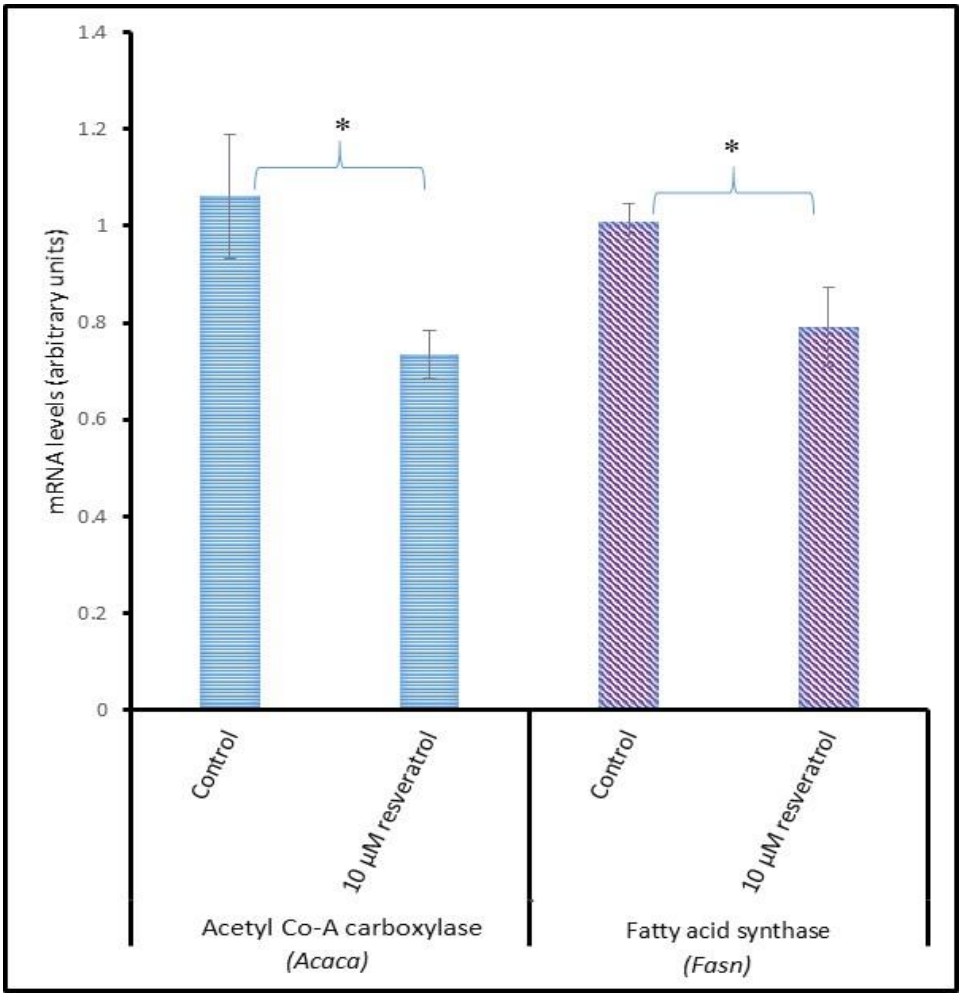

Figure 6. 2: The effect of resveratrol (10 µM) on acetyl Co-A carboxylase (ACC) and fatty acid synthase (FAS) mRNA applied between days 10 to day 12 after inducing differentiation in 3T3-L1 cells. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate. Cells were treated with resveratrol (10 µM) from day 10 to day 12 for 48 h. Total RNA was extracted, and mRNA levels of FAS and ACC were measured by RT-qPCR. Data are expressed relative to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and normalised to control. Values are means (n=12), with standard errors represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. *P≤0.05.

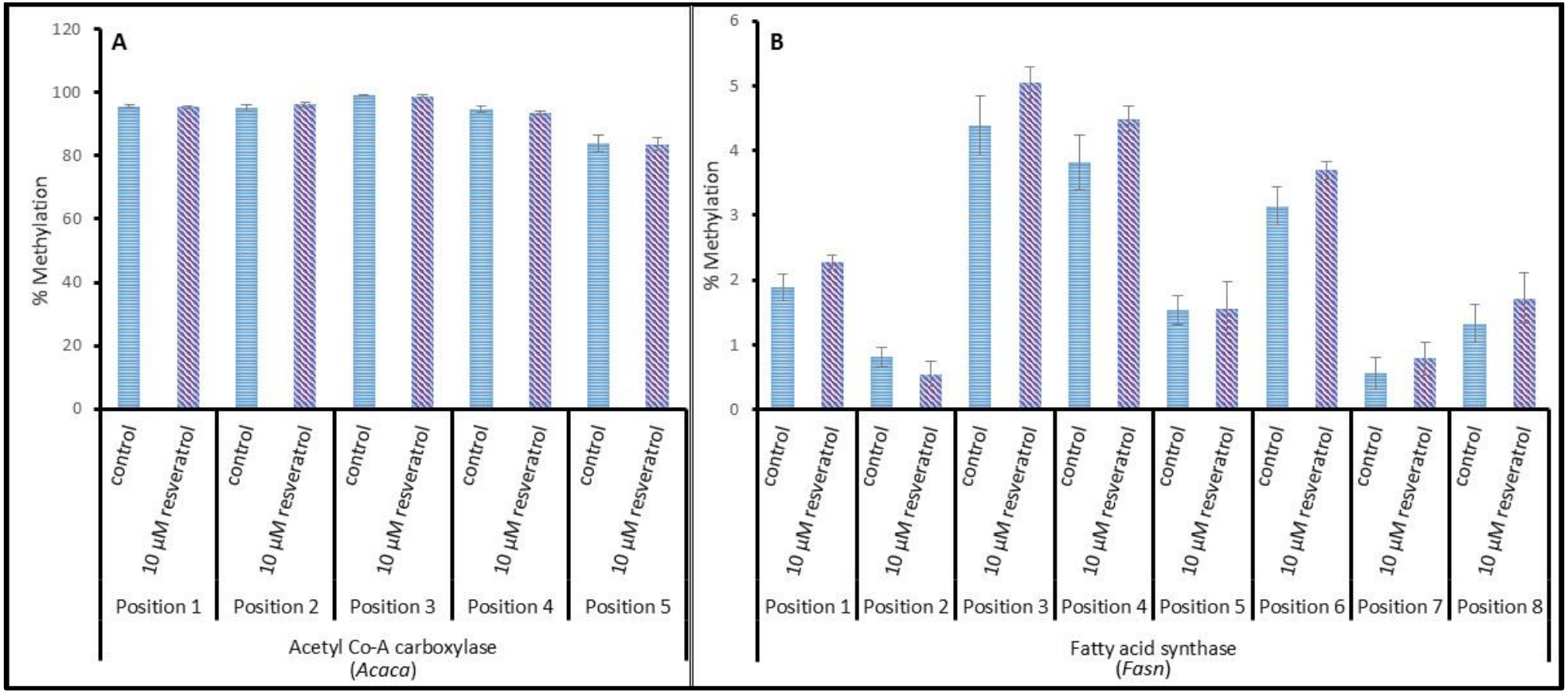

Figure 6. 3: The effect of resveratrol (10 µM) applied between days 10 to day 12 after induction of differentiation on DNA methylation at CpG sites in acetyl co-A carboxylase (ACC; Acaca) (panel A) and fatty acid synthase (FAS, Fasn) (panel B) gene promoter regions in 3T3-L1 cells. Cells were seeded and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate and treated between day 10 and day 12 for 48 h. Genomic DNA was extracted on day 12. DNA methylation at specific CpG sites was measured by pyrosequencing. Values are means (n=12), with standard errors represented by vertical bars. Statistical analysis was by Student`s unpaired T-test. P> 0.05.

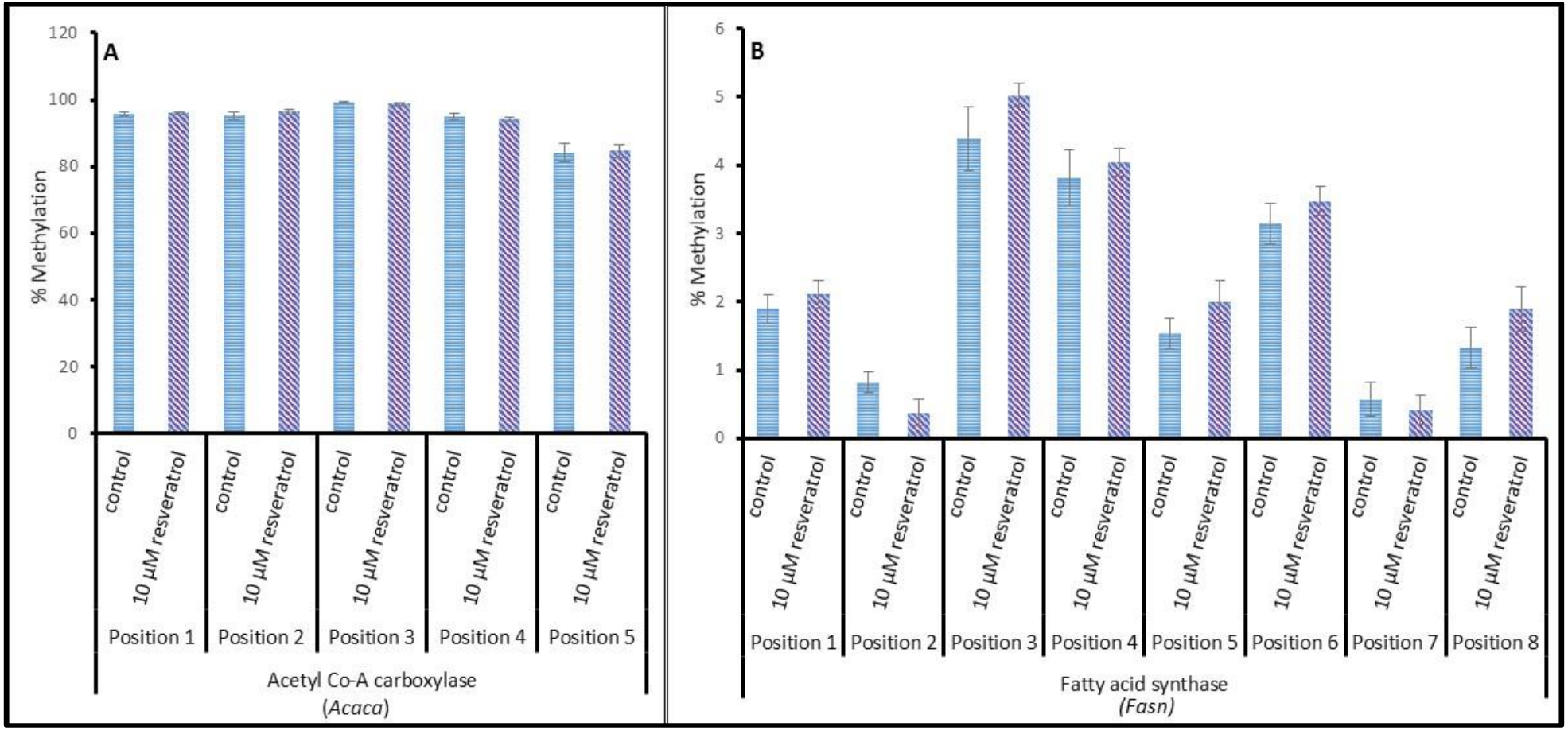

Figure 6. 4: Effect of resveratrol on methylation status of acetyl co-A carboxylase (ACC; Acaca) (panel A) and fatty acid synthase (FAS; Fasn) (panel B) promoter applied on induction of differentiation in 3T3-L1 cell. Cells were cultured and grown to confluence in 6-well plates. Forty eight hours after cells reached confluence, taken as day 0, cells were induced to differentiate in the presence and absence of resveratrol (10 µM). Genomic DNA was extracted on day 12. Assays measured percentage of DNA methylation at different CpG sites as indicated. Values are means (n= 11-12), with standard errors represented by vertical bars, compared by Student`s T-test. P>0.05.

6.4. Discussion

In this chapter of the study I investigated the effect of resveratrol on DNA methylation of the genes coding for acetyl Co-A carboxylase (*Acaca*) and fatty acid synthase (*Fasn*), which are the key and rate limiting enzymes in the process of lipogenesis (Hellerstein et al., 1996; Ameer et al., 2014), We hypothesised that altered DNA methylation may be the mechanism underlying a previous observation made in the laboratory by Goiuri Alberdi Aresti, which was that resveratrol reduced the mRNA of these lipogenic enzymes in 3T3-L1 cells. This hypothesis was informed by previous studies where resveratrol was shown to regulate expression of genes via inducing changes in DNA methylation (Melzner *et al.*, 2002; Stöger, 2006). I thus measured DNA methylation at 5 and 8 sites in *Acaca* and *Fasn* promoter regions, respectively, before and after treating 3T3-L1 cells with resveratrol.

I first measured DNA methylation of *Acaca* and *Fasn* promoters before (day 0) and after induction of differentiation (day 12), and observed that DNA methylation of neither gene at the CpG sites measured changed when cells differentiated. The purpose of making these measurements was to be able to take into account any effects of resveratrol I might measure that were commensurate with effects on cell differentiation status. However, the fact that DNA methylation of the *Acaca* and *Fasn* genes remained constant throughout the differentiation process indicated that DNA methylation may not be an important factor in controlling the expression of the *Acaca* and *Fasn* genes, since both genes were observed to undergo large changes in expression as 3T3-L1 cells differentiate (see chapter 3, section 3.3.1). Thus this initial observation suggested that DNA methylation may be an unlikely mechanism underlying the effect of resveratrol on expression of these genes.

Indeed, resveratrol did not affected DNA methylation of Acaca and Fasn, yet in agreement with the previous work in the laboratory it reduced the corresponding mRNAs. Observations made in vivo have also shown a lack of action of resveratrol on DNA methylation of the Fasn gene. A dietary supplement of 30 mg/kg/day in rats was found to have no effect on *Fasn* promoter DNA methylation, yet it was effective in protecting against diet induced obesity (Gracia et al., 2014).

To the best of our knowledge, this study is the first to investigate the effect of resveratrol on *Acaca* DNA methylation. The CpG content of the *Acaca* promoter is very

low and does not satisfy the following criteria for defining the CpG island: region > 200 bp; CpG content ≥ 50% (Deaton and Bird, 2011; Elango and Yi, 2011; Du *et al.*, 2012). There is a general view that promoters that contain CpG islands are regulated by DNA methylation whereas it is a less important mechanism for controlling the expression of genes that lack CpG islands (Du *et al.*, 2012; Rao *et al.*, 2013). However, DNA methylation of genes with no CpG islands seems to have a role in controlling gene transcription (Han *et al.*, 2011; Øster *et al.*, 2013). Thus I considered it still worth investigating if resveratrol affected DNA methylation of the *Acaca* promoter.

The effect of resveratrol on *Acaca* and *Fasn* expression may be through inhibiting the expression of sterol response element binding protein-1c (Srebp-1c) and PPARγ, and subsequently their downstream lipogenic genes comprising *Fasn* and *Acaca* (Lin *et al.*, 2012b; Jin *et al.*, 2013; Andrade *et al.*, 2014a), up-regulation and activation of Sirt1 (Qiang *et al.*, 2012; Andrade *et al.*, 2014a), which has also been shown to affect the expression and activity of FAS and ACC (Cheng Xu *et al.*, 2013; Zhang *et al.*, 2014)

In summary, changes in the DNA methylation at the CpG sites measured by in our assay appears to be not an important factor in the controlling the expression of *Acaca* and *Fasn* genes as neither the differentiation process nor resveratrol treatment induced changes in DNA methylation at these sites. Thus, future experiments to explore the role of DNA methylation and the basis of the action of resveratrol to reduce *Acaca* and *Fasn* mRNA in 3T3-L1 cells could measure different CpG sites in the promoter and enhancer regions of these genes or of their upstream transcription factors. Additionally other mechanisms such as changes in transcription factor expression should be investigated

Chapter 7

7. Summary discussion and Conclusions

The consumption of dietary polyphenols, including resveratrol and genistein, has been encouraged recently with the aim of deriving health-beneficial effects. A wide range of possible health benefits related to consuming resveratrol and genistein have been shown in numerous epidemiological and experimental studies. The possible positive actions include anti-cancer activity (Wang *et al.*, 2002; Sales and Resurreccion, 2013), antioxidant effects (Hoshino *et al.*, 2010; Sales and Resurreccion, 2013), and antilipogenic activity (Kim *et al.*, 2010; Jin *et al.*, 2013). This study aimed to investigate certain actions of these dietary polyphenols in relation to aspects of molecular physiology relevant to metabolic health and aging, and to explore the possible mechanisms of such actions. The dietary polyphenol genistein was the main focus of this study.

Genistein appears to have pleiotropic cellular actions mediated through different molecular targets, and it is difficult to attribute physiological effects to specific actions at the molecular level. The main focus was its possible use as an agent to reduce adipogenesis. Related benefits would include alleviation of obesity-related health problems such as cardiovascular disease, type-2 diabetes and non-alcoholic fatty liver disease.

Previous research in the laboratory using the mouse 3T3-L1 adipocyte model showed that resveratrol affected the expression of genes involved in lipogenesis, specifically *Acaca* and *Fasn*. I proposed that genistein, which has some structural and possibly functional similarity to resveratrol, would have a similar action. However, addressing this hypothesis led to the serendipitous discovery that genistein had dose and time dependent effects on the appearance of brown/beige rather than white adipocytes as indicated by accumulation of small paucilocular lipid-droplets within the cytoplasm of genistein treated cells, which opposed the typical large fat droplets observed in the control differentiated cells. Consistent with this morphological change there was a decrease in the expression of the mRNA levels of genes that characterise white adipocytes, specifically *Fasn, Fabp4, Lipe, Rarres2 and Retn*. A notable exception to this pattern of gene responses consistent with genistein promoting a beige adipocyte gene expression profile was *Acaca*, which initially increased after the application of a high dose of genistein but was ultimately

(like the other markers of white adipocytes) down-regulated. The metabolic rationale for this observation is not clear, and a search of current literature did not reveal any evidence that ACC has a specific role in white into brown adipocyte interconversion. Thus, probing the role of ACC may be an interesting and important objective for future study to understand distinct metabolic activities of the different types of adipocyte tissue. As expected, concomitant with a reduction in the expression of genes that characterise white adipocytes, there was an increase in the expression of genes involved in the process of white into brown adipocyte interconversion *(Sirt1, Cebpb, Ppargc1a)* accompanied by an increase in the expression of genes that characterise beige adipocytes (*Ucp1* and *Tnfrsf9).* Uncoupled mitochondria, due to expression of UCP1, are a key phenotypic feature of brown and beige adipocytes. In parallel with a marked increase in UCP1 expression observed in 3T3-l1 cells in response to genistein there was an increase in basal and uncoupled oxygen consumption rate. Furthermore, the proportion of the total oxygen consumption rate that was uncoupled before addition of uncoupling agent FCCP and after activation of UCP1 by the retinoid TTNBP was increased. Together these findings are consistent with increased uncoupling of mitochondrial oxygen consumption from ATP synthesis and thus with the induction of a beige adipocyte phenotype.

These observations suggest that genistein, which is present in a large quantities in soya-based products (e.g. including tofu, fermented bean paste and soya milk), may protect against obesity by enhancing the development of brown, rather than white, adipose tissue. However, to substantiate such claims it is essential to demonstrate these effects in human volunteers. There are many other limitations and caveats to this work, which should be addressed in future studies. Firstly, these observations were made after direct exposure of 3T3-L1 cells to genistein, which is not achieved when genistein is consumed in the diet. The parent compound genistein, rather than its metabolites, may be the active stimulator of the process of white to beige adipocyte conversion. Thus further work should investigate if genistein is metabolized in this cell culture system, and should determine if individual metabolites of genistein, which are commercially available, evoke the same response.

The fact that I derived beige adipocytes from 3T3-L1 cells provides further evidence to support published reports that beige adipocytes originate from the same precursor as

white adipocytes, which is different from that of classical brown adipocytes. Further work is required, however, to determine if the process of interconversion is transdifferentiation of mature white adipocytes or de novo differentiation of precursor cells induced by genistein. The in vitro system affords the opportunity to address this fundamental question through approaches such as genetically marking the mature white adipocytes (for example, with β-galactosidase under the control of white-adipocyte-specific promoter) then observing the distribution of marked cells after induction of the phenotypic change by genistein.

Genistein has structural similarities with 17β-estradiol and is, therefore, described as a non-steroidal phytoestrogen. Thus, I determined if the responses I observed in 3T3-L1 cells were mediated via the ER. This was achieved by determining the effect of the ER antagonist fulvestrant on the observed responses to genistein. The results indicated that browning of white adipocytes is probably not mediated through the ER as the observed change in the morphology of cells induced by genistein was not suppressed and the changes in expression of our test gene panel was not abolished by fulvestrant.

Changes in Sirt1 expression occurred early in the sequence of effects I observed, and previous studies demonstrated a likely role of Sirt1 in mediating browning of white adipocytes (Qiang *et al.*, 2012; Fu *et al.*, 2014). Thus, I proposed that Sirt1 activity may be a key initiating step in the process of white into beige adipocyte interconversion induced by genistein. In support of this hypothesis, the responses of *Ucp1* and *Cebpb* genes to genistein were significantly attenuated when the activity of Sirt1 was inhibited by EX-527, which is a specific and potent Sirt1 inhibitor with the ability to bind in the nicotinamide pocket of the Sirt-1 protein (Gertz *et al.*, 2013). Also, the genistein-induced reduction in the expression of genes that characterise white adipocytes (*Acaca, Fasn, Fabp4, Lipe, Rarres2 and Retn*) was not attenuated by EX-527. From these observations I conclude that Sirt1 activity is a component of the process that initiates white to beige adipocyte conversion. I also showed a moderate (2 fold) increase in Sirt1 mRNA and a sharp increase (approximately 12 fold) in Sirt1 protein, which further indicated that increased Sirt1 action was a vital mediator in the observed changes.

The effect of genistein on Sirt1 expression was also shown to not be unique to adipocytes. Genistein also was found to promote an increase in the expression of Sirt1 in

MCF-7 breast cancer cells and the effect was ER-independent, similar to the observations made in 3T3-L1 cells. The role of Sirt1 in promoting white into beige adipocyte conversion could be further investigated by targeting Sirt1 with other potent pharmacological or dietary agents, which may allow a better understanding of this process, and may ultimately allow the development of new dietary or pharmacological strategies to promote beige adipocyte tissue development and thus protect against metabolic diseases.

To develop previous work conducted in the laboratory on novel actions of resveratrol (Alatawi, 2012), we proposed that genistein like resveratrol would affect histone expression. Histone proteins control gene expression (Chen *et al.*, 2013a) and may influence life expectancy (Feser *et al.*, 2010a; Hu *et al.*, 2014). We also proposed that genistein may have a synergistic effect on histone expression when combined with resveratrol. Lower reporter gene expression was measured in extracts from Caco-2 cells transfected with a histone H3 (variant H3b) promoter-reporter construct after treatment of cells with resveratrol and/or genistein. The effect of resveratrol and genistein in combination, however, was not greater than that seen with either compound separately, indicating that the compounds probably affect histone H3 gene transcription through the same mechanism but don't act in synergy. However, in this study an interacting effect of genistein and resveratrol was tested only with respect to a very selective action. Thus, I should not exclude the likelihood that these compounds interaction over a broader spectrum of actions. Interaction of genistein with resveratrol and other dietary polyphenols would be worth investigating more broadly.

The effect of genistein on histone protein abundance contrasted with our observations that genistein repressed transcription from the histone H3.1 (variant H3b) promoter-reporter construct. Genistein induced an increase in the expression of histone H3 and H4 proteins in both Caco-2 and MCF-7 cells. I suggested that genistein might have differential actions on specific histone variant mRNAs. In support of this hypothesis, histone H3d and H3.3 mRNAs were found to increase in response to genistein whereas all other variants measured were reduced. Again, the ER antagonists fulvestrant and G15 did not affect any of these responses, indicating that, like the other actions studied in this body of work, these effects of genistein were though pathways independent of ERs. Observations of these ER-independent actions of genistein on histone expression

extended also into 3T3-L1 adipocytes, the main focus of the body of work in this thesis. The significance of this finding with respect to cell physiology is not yet clear. However, gene expression through specific alterations in chromatin structure may be one of the mechanisms through which genistein has its reported pleiotropic beneficial effects. Specific and distinct roles of histone H3 variants have been shown; thus the findings are likely to be important. For example, the presence of histone H3.3, especially at specific gene promoter and enhancer regions, was associated with transcriptional activity (Ahmad and Henikoff, 2002; Hake and Allis, 2006; Chen *et al.*, 2013a). However, to establish an association between a genistein-stimulated increase in the level of histone proteins and actions of genistein on gene expression further study is needed. For example, histone proteins could be enriched using chromatin immune precipitation (CHIP) then their associated genes could be detected using PCR. Furthermore, the observed stimulatory effect of genistein on histone protein abundance may promote longer life expectancy (Feser *et al.*, 2010b; O'Sullivan *et al.*, 2010). To address this hypothesis work could include investigating the effect of genistein on lifespan and histone expression in animal models.

Because of the structural similarities between genistein and 17β-estradiol I investigated if genistein affects the expression of the ER, since 17β-estradiol has autoregulatory feedback action via repression of ERα expression. The observations made were potentially important because I showed that genistein has selective actions on ERα versus ERβ, reducing mRNA corresponding to ERα while increasing ERβ mRNA. Once more, these responses were not affected by ER antagonists fulvestrant and G-15. The down-regulation of ERα concomitant with an increase in the expression of ERβ may be physiologically beneficial. ERα activity is generally detrimental while ERβ activity appears to be of benefit in the prevention or treatment of breast cancer (Stettner *et al.*, 2007; Hartman *et al.*, 2009; DeSantis *et al.*, 2011; Liao *et al.*, 2014). The potential of genistein to reactivate the expression of ERβ in ERβ-negative cancer patients may have therapeutic potential for the prevention or treatment of hormone-dependent cancers prior to and during hormonal therapy.

Finally, I developed previous research in the laboratory on actions of resveratrol in 3T3-L1 cells. The earlier work showed that resveratrol reduced mRNA levels of genes involved in the initial steps of lipogenesis, specifically *Acaca* and *Fasn*. I reconfirmed this

finding in the work presented in this thesis. We proposed that this action may be accompanied by a change in DNA methylation of the corresponding gene promoter regions, which is a plausible mechanism of regulation given the other reported effects of resveratrol to alter DNA methylation. However, resveratrol had no effect on DNA methylation in the regions measured. Thus, this initial observation suggested that DNA methylation may be an unlikely mechanism underlying the effect of resveratrol on expression of these genes.

Together the body of work presented in this thesis uncovers novel actions of the dietary polyphenol genistein, particularly in reaction to adipocyte tissue browning, but also more broadly, encompassing action on histone and ER expression in adipocytes but also other cell types. Our findings indicate that genistein mediates white to beige adipocyte interconversion. This action may, in the context of a favourable background diet, aid in the prevention of obesity and its related disorders including cardiovascular diseases and diabetes. In addition, a differential regulatory effect of genistein on ER expression that I uncovered may be of benefit in the prevention and/or treatment of hormone-dependent cancers. Alteration in chromatin structure driven through differential effects on the expression of specific histone variants is one of the possible mechanisms through which genistein may have its pleiotropic actions on gene expression. Despite the structural similarity between genistein and the endogenous ER ligand 17β-estradiol, I showed that all of the actions of genistein were mediated through ER-independent mechanisms.

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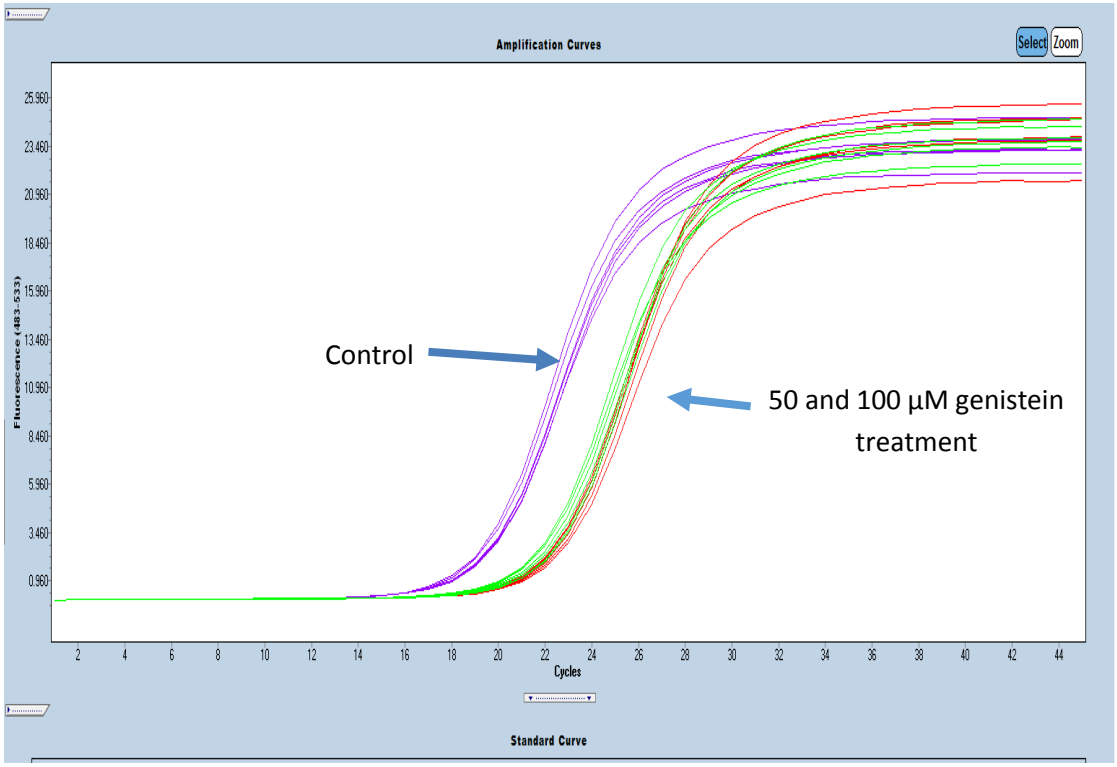
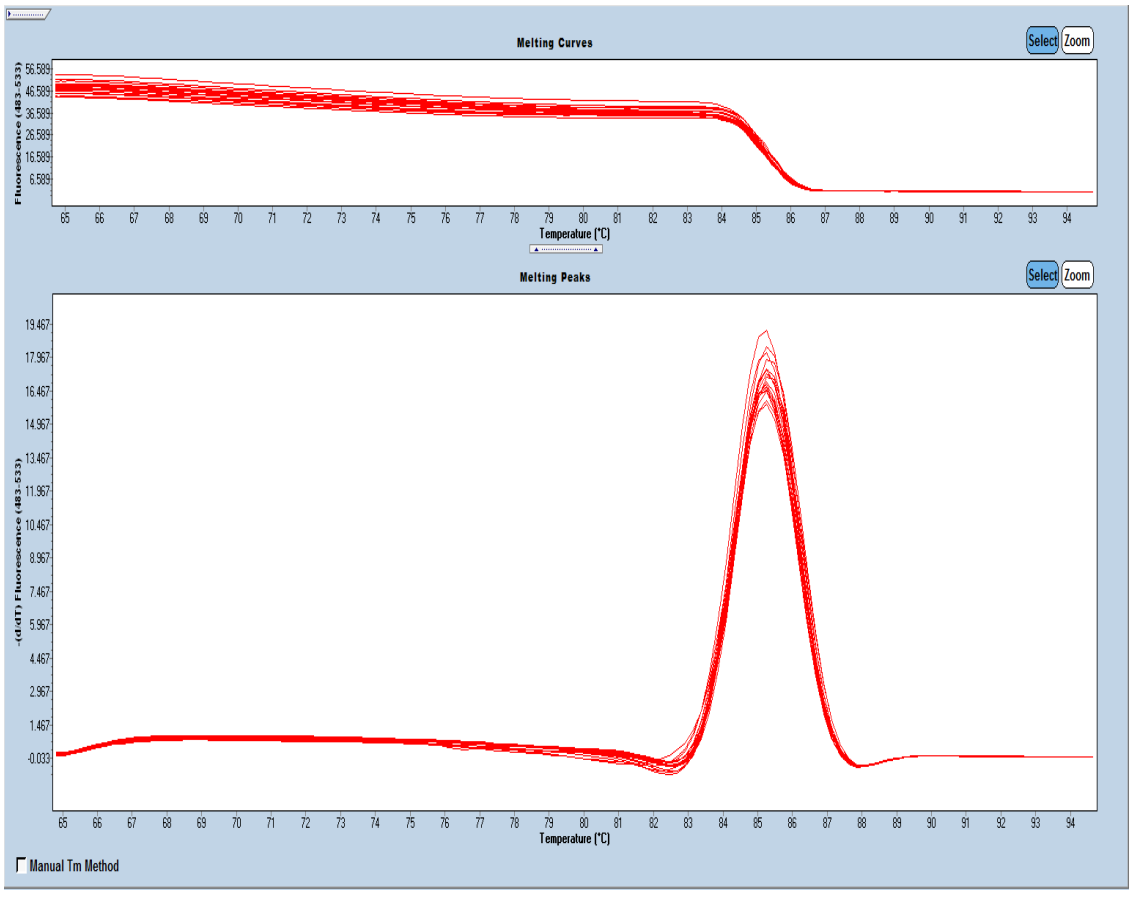
Appendix 1: Representative amplification curves, melting curves and melting peaks for RT-qPCR.



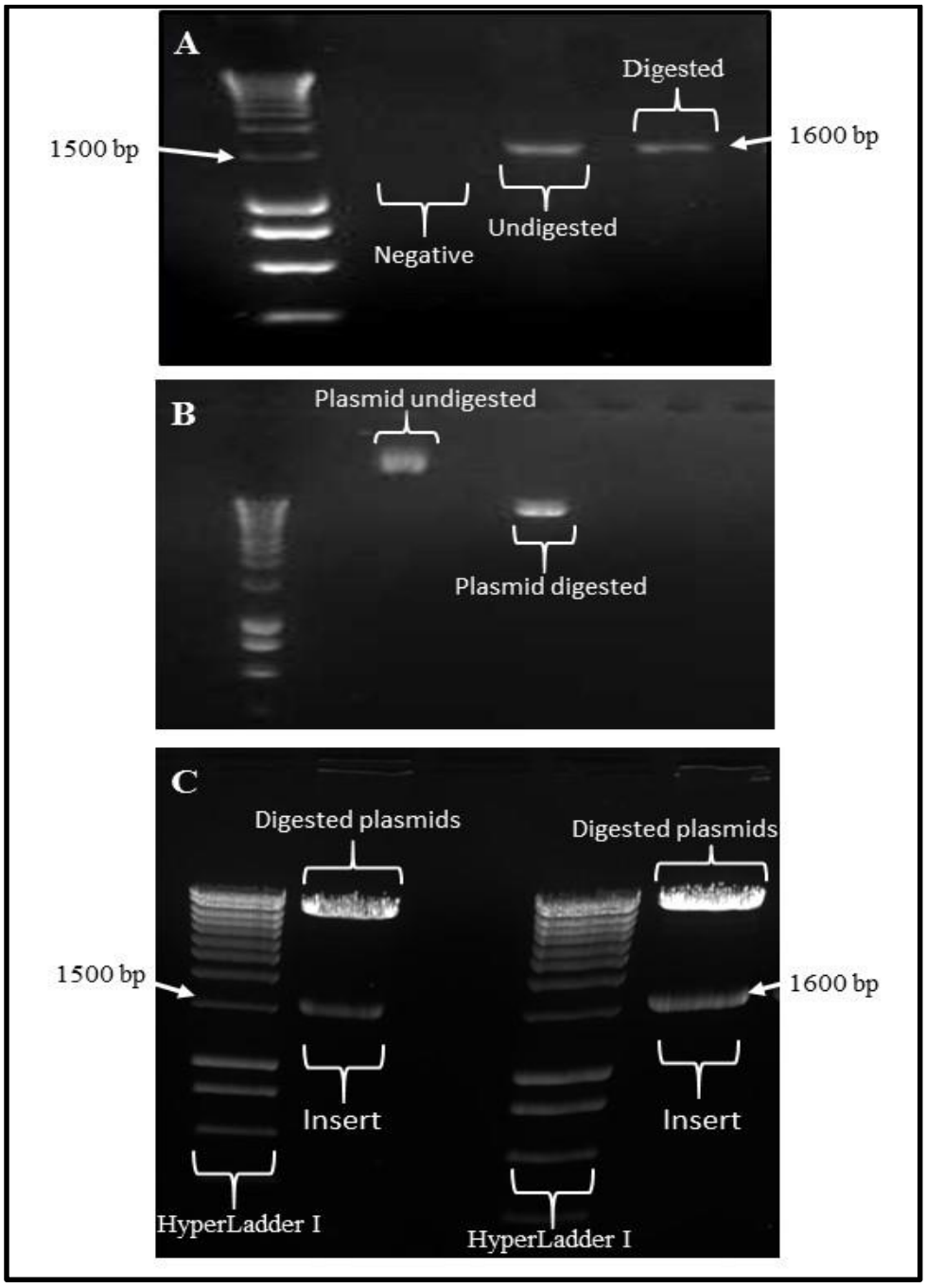


Appendix 2: Histone H3b and H3d promoter alignment

```
H3d      TATGAAACC------AATTTGACA----------------AGGGACACATCACAGCACAA
H3b      TGTGGCACTTTTAGTAATTTGTCCTGCAGAAATTAGATCCATAGAAAC--CTCAGGAATT
         *.**..**       ******:*.                * .**.**  *:*** *.::

H3d      -----CATAAGAAAAATGTGCTTATCAGCACAATGGGCTAGCCTTCTAACCAGCACTTGG
H3b      CTAGGTATGTGGGAGAAGTGCCATGCAGCACAAAAC--ATG-----TTT-----AC-AGG
               **.:*..*.*:**** :: ********:.   ::*     *::     ** :**

H3d      GGTAAAACA----------AACATG---------CTACGGTTAAGT--ATAGCAT--TTT
H3b      GGTGATTCGCGTTAAGTTTCACACACAGCAGTTACTACATTTTAGAGGAAGGAAATTATA
         ***.*::*.          .*** .         ****. **:**:  *:.*.*:  :*:

H3d      CC-T----------TCAACCAGTCAAAAGAACTGTGGTCTAGACCTACACAGCAGCAACC
H3b      CCCATGAGTGCATTCCTAACTATCTT--GAAT---GGAAGTGT---TAAAACCCGCATGC
         ** :           *:*.*:.**::  ***    **:. :*:   :.*.* *.***: *

H3d      CCCTCAAATG--AGGATAAAACCAAACCTGTTGGT------------GGTAAACACGGAA
H3b      CCCACACAAGTTTGAAT-ATGTCATACCATTTGCTGTAGCAATTAATGGCATACACAATT
         ***:**.*:*  :*.** *:. **:***: *** *            ** *:****..::

H3d      AATATGCAGGTAATTACAACTCTATAGCTGAAAAAGTGGGT----GGCT-CTTAAAAGAG
H3b      -----GAGAGCACACAC---ATTACCACTGAACATTTGAGTATGTATTTCCCAAAATGAG
              *...* *.: **   : ** ..*****.*: **.**    .  * * :***:***

H3d      CCTTTG---GGATTGGGTATGAAGACGTTAGAATTACTTAGAGCTGGTGTACTTGGTGAC
H3b      CTTTTTTCCAGTTTGGGGAT----------GTTTTGCTTTGTTTTGGGG----TGGAGTC
         * ***    .*:***** **          *::**.***:*:  *** *    ***:*:*

H3d      GGCCTTGGTGCCCTCTGACACGGCGTGCTTAGCCAGCTCCCCGGGCAGCAGCAGGCGCAC
H3b      ---------TCCCTCTCGCCCAAGCTG-------------CAGTGCAGCGGCGTGATAAC
                   ****** .*.*..  **             *.* *****.**. *. .**

H3d      GGCCGTCTGGATCTCCCTGGA--GGTGATGGTGGAGCGCTTGT--------TGTAAT---
H3b      AGCTCACTGTAACCTCGAACTCGGGCTCAAGCGATCCTCTTGACAGCCTTCTGAGTAGCT
         .**  :*** *:*  * :. :  **  .:.* *.: * ****:        **:.::

H3d      ---GCGCCAGGCGG--GAAGCCTCGCCAG-CGATGCGCTCGAAGATATCGTTGACGAAGG
H3b      GGGATTACAGGCGAGAGCCGCCACGCCCGGCTAAGAGCATT-------------------
            .  .******.  *..***:****.* * *:*.**:

H3d      AGTTCATGATGCCCATGGCCTTGGATGAGATGCCGGTGTCGGGGTGGACCTGCTTTAGCA
H3b      -TTTCTAATTGCCCACA-CTTCTTATGCGA----------------------------CA
           ***::.:****** . * *   ***.**                            **

H3d      CCTTGTACACGTACACGGAATAGCTCTC--CTTGCGGCTACGCTTGCGCTTCTTACCATC
H3b      CCCA-------------GAAAAATACAATTTTAAATAAAGCGCATATGCAAATTTCC---
         ** :             ***:*. :*:.   *:.. ..:.***:*. **::.**:**

H3d      CTTCTTCTGCGCCTTG-GTCACCGCCT---TTTTGGAGCCCTTTTTTGGAGCAGGA-GCG
H3b      -CTAATCGTCTCCAATATTCTCTGATTTCTTTTTTATATTTTAACTAGAAACAATTGGAG
           *.:**  * **::   **:* *. *   **** .:.   *:: *:*.*.**. : *.*

H3d      GA-----CTTAGCAGGTTCAGGCATGATAAATAAACACT--------------------
H3b      GTTTCCGCGTTGCTTTGTGTGGTTGTAAATTTTAAGACTTCAGGAAACTTTTCCAGTACA
         *:     * *:**:   * :** :  *:*::*:** ***

H3d      ----TCGTCCACAACCT-----------AAAGAGAAATGCAAGTGTCCTCACTCTGCAGC
H3b      AGACTTGTCCACAGTGGATATAGCAGCTAAGGGGTTAACAAAATGACGTCAG--AGTAGC
```

```
            * *******.             **.*.*::*: .**.**:* ***   :* ***

H3d      TGGTAATATTTGTAGAGCTCCTATCGTAAACTGGCATTTAAAAGCTTACACATTGATTGG
H3b      TACGG-TAATGGGCAGGAGCCTCTCTTAATCTGC-------AACCAGGCACAGAGATGGA
         *.  . **:* * ...*. ***.** ***:***        ** *: .**** :*** *.

H3d      ATGAATTGTCTCT-GTGTGCCCTGTGGTATGCAAATTAGGTGATTCTAGCGTTTCTTTGC
H3b      ----CCAATCCAAGAAGGGCGCGGGGAT------TTTTGAATTTTCTTGGGT-------C
             . :.** .: .:* ** * * *.*      :**:*.: :****:* **       *

H3d      AATTGGTTGGTTGTTCTAGCC-AATCA-AATAGCGTT--ATTTACAATTCGACCTCCAAG
H3b      CAATAGTTGGTGGT-CTGACTCTATAAAAGAAGAGTAGCTCTTTCCTTTC---CTCCA--
         .*:*.****** ** **..*  :**.* *.:**.**:  : **:*.:***   *****

H3d      TATAATAGTTTCTCACTTGCTAACAAAACTCACTTTTACATTTTTGTCTTCATTGCTTAA
H3b      ----------------------------------------CAGACGTCTCTGCAGGC
                                                  :*:* **: ***: ..
```

Appendix 3: (A) Agarose gel electrophoresis of human ERβ PCR products and restriction digestion. Human genomic DNA was used to amplify about 1600 bp of ERβ promoter region and restriction enzymes digestion by Hind III and EcoR1. (B) Agarose electrophoresis gel of the plasmid (pSF-pA-PromMCS-BetaGal vector) before and after restriction enzymes digestion by Hind III and EcoR1. (C) Agarose gel electrophoresis of recombinant plasmids to screen for those containing the ERβ promoter region. The DNA molecular weight marker (hyperladder I) was run in parallel to monitor the size of products.



Appendix 4: Acaca and Fasn promoter sequence before and after bisulfite modification are shown.

Acaca gene promoter DNA sequence:

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TTTTTGGAGGCGGATATCTGCTGAGACAATAAGAATTATAAGAGGTCAGTTTCAAATAATAGTCTTTTATGT
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TTGGCAGACGTAGGGTGAGTGACCCAAGCGAACAGGGAGGCTGACAATGACAACGGTGGTGACCCACTGCCT
TCTCTCTAATTTAGGAC**TGGAATCAATGGAAATGGGACAGG**CTGTGGCAGCTTCTAGAGTGTCTGGATGGCT
ACTCAGTGATTTTCCAGGCATGACCTCCCTTTCTGGTGGCATACATGCCTGCGCTAGGGAGCAGTGTTCCGT
ACAGGTGCTTGCCGCTGGGCAGGCGTCAGTTGTTAAACACGGCTGCTTCCAGTCTGTGCAGCCCTTAGAAAA
GGCTGCCTGGGTTCTGGCCGCTAGGAGGGGCCAGGATGAAGGGCTGTGCTGAGCCTTTCATTCATCAGCCCA
GGGACTGTATTTTACTAACGCTGACCTTCTTTACTTTCATGATCTCCAAGCGTCACTACTTTCTCATGAACT
TTATTTTATTTTTGTGACCATCAGATTTCAGTCAGATTATAAATTAGAATGTACACCTGTGTGCCTTGGTTC
CCTTGTGAAGGCAGCAGCTGTGAGCCAGGTGTGGAGGTGTACTAATGTCATCCCAGCTATAGGTATCCCCTG
CTGTAGAAGTTTGGGGTTAGCCTGACTGTGAGATGCTGTCTCTTCCCTGCCTCTCCAAATACTTGGCCAGCT
TTCATTTTTTTATTCTCTCGGTTGCTGTATCTTTTATTATTTTTAACATGGTCAGTGTATTGGAATGTCACT
TTTTCCTTTCCTCTTATTCCCTAACTCAATTTTTCTTTTTTTTTTTAAGATTTATTTATTTATTTTATGTAT
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TCCCTGTCACCAGCCTCCGTCAGCTCAGATACACTTTCTGATTTGGGGATCTCTGGCTTACAGGATGGTTTG
GCCTTTCACATGAGGTGAGAAGAA

Bisulfite modified *Acaca* gene promoter DNA sequence:

TAATATTATTAATTTTATTATTTTTGTATTAGATTGAGTAGAATTGGAATTGAGAAAAAAAATTGGTTTTTA
AAAGAAGAAAAAGAAAATTAAATGAATAGAAAAAGTAATTTTTAAATTTTTGTTTTTTTTTTTATTTAGTTT
TTTTTGGAGGC/TGGATATTTGTTGAGATAATAAGAATTATAAGAGGTTAGTTTTAAATAATAGTTTTTTAT
GTTTATATTGTAGTAAAAGTTTTAATGTGTTAAGATGTTAAATTGTGGGTTTTTGTTGTTTTTTATAAAGAA
TATTGGTAGAC/TGTAGGGTGAGTGATTTAAGC/TGAATAGGGAGGTTGATAATGATAAC/TGGTGGTGATT

TATTGTTTTTTTTTTAATTTAGGATTGGAATTAATGGAAATGGGATAGGTTGTGGTAGTTTTTAGAGTGTTT
GGATGGTTATTTAGTGATTTTTTAGGTATGATTTTTTTTTTTTGGTGGTATATATGTTTGC/TGTTAGGGAGT
AGTGTTTC/TGTATAGGTGTTTGTC/TGTTGGGTAGGC/TGTTAGTTGTTAAATAC/TGGTTGTTTTTAGTT
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Fasn gene promoter DNA sequence:

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GCGGCCTAGAGGGAGCCAGAGAGACGGCAGCGGCGTCCCGTCCAGTTCGCCTGCCGCGCTCCTCGCTTGTCG
TCTGCCTCCAGAGCCCAGACA

Bisulfite modified *Fasn* gene promoter DNA sequence:

TGGTTGGTTTATATGGTGGGTTATTATAATATTATAGGGAAGTTTATATTTAGTAATGTGGTTTTGGATGTT
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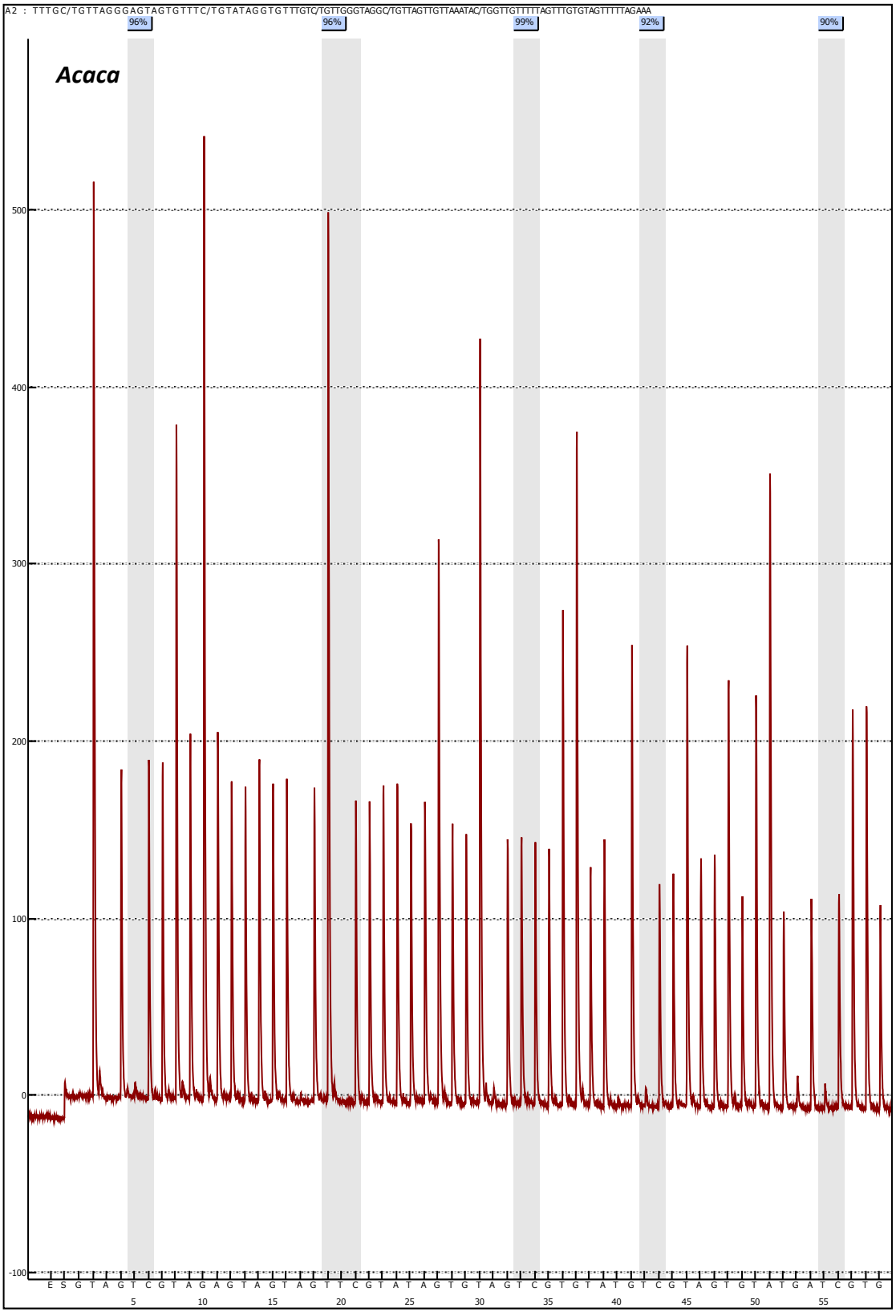
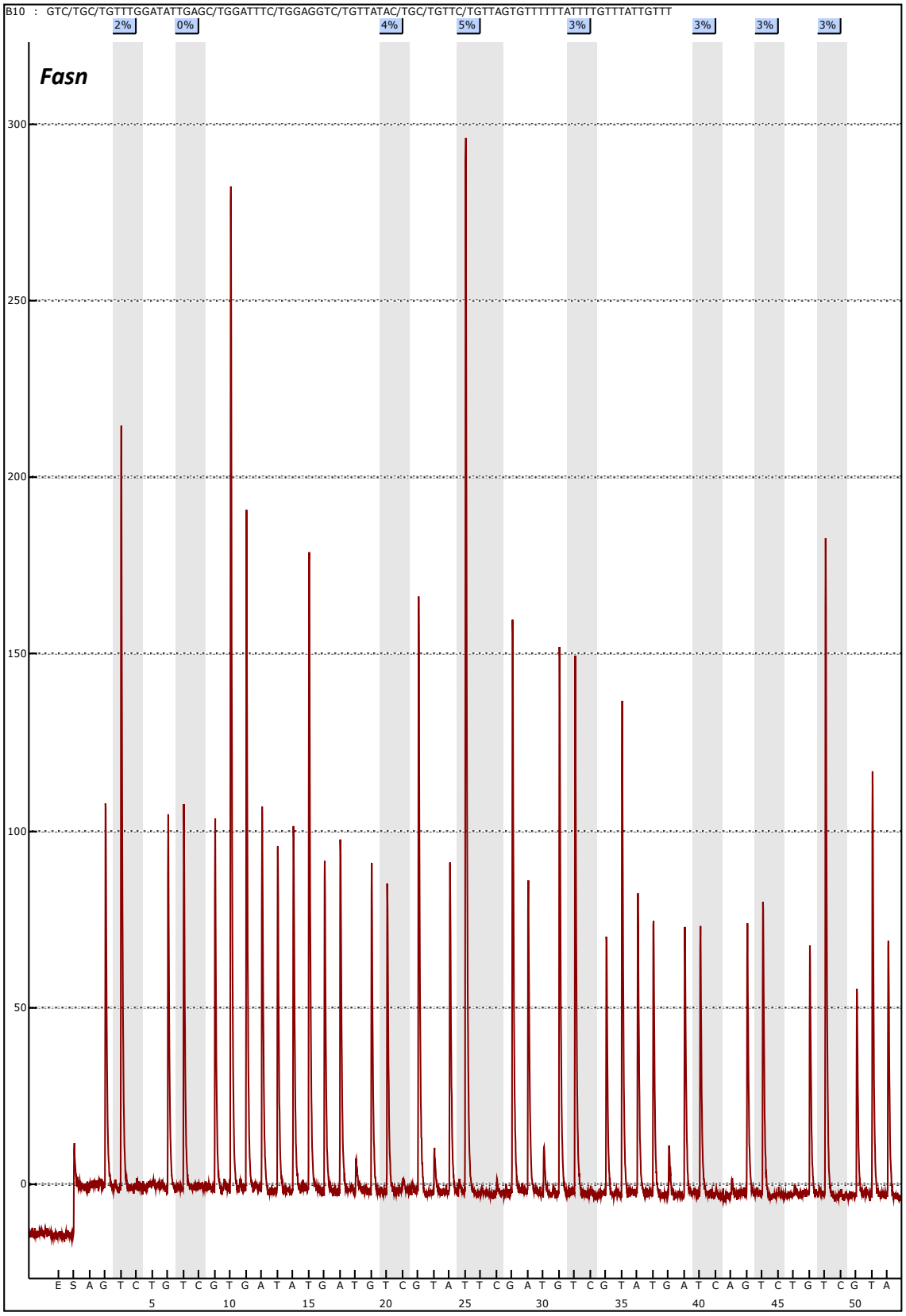
Appendix 5: Representative pyrogram of Acaca and Fasn

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2%
0%
4%
5%
3%
3%
3%
3%
Fasn
300
250
200
150
100
50
0
E S A G T C T G T C G T G A T A T G A T G T C G T A T T C G A T G T C G T A T G A T C A G T C T G T C G T A
5
10
15
20
25
30
35
40
45
50