Obesity and Vitamin D3 supplementation as modulators of tumour development and the immune microenvironment

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

By

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Abstract

Obesity and Vitamin D3 supplementation as modulators of tumour development and the immune microenvironment

Considerable recent attention has focused on obesity as a major global health problem. Obese individuals are at increased risk not only for metabolic syndrome, but also for different types of cancer including melanoma. The consequences for melanoma tumour development was explored with a high fat diet (HFD) which induced obesity in a Low-density lipoprotein receptor deficient ($LDLR^{-/-}$/C57BL/6) mice. Additionally, the presence or absence of dietary Vitamin D3 supplementation (in HFD fed mice) on melanoma tumour growth was examined. *In vitro* assays demonstrated the key role that fatty acids (FAs) (either purified or present in the serum of HFD fed animals) had on the properties of B16-F10 and macrophages. FAs and serum from HFD fed mice fuelled B16F10 melanoma growth and led to an increase in cell proliferation, migration, lipid inclusion and altered chemokine production. Furthermore, FAs also enhanced expression of pro-inflammatory mediators by macrophages. VitD3 affected B16-F10 in a dose dependent manner. However, VitD3 supplemented HFD serum increased B16-F10 proliferation. In the *in vivo* part, $LDLR^{-/-}$ mice were subjected to different types of diet (HFD, VitD3 supplemented HFD and control diet) for two or ten weeks prior to syngeneic implantation of B16-F10. After two weeks post-injection of B16-F10, HFD feeding increased tumour growth combined with increases in adipose tissues in $LDLR^{-/-}$ tumour bearing mice, induced inflammation; modulated expression of specific receptors implicated in obesity (GPR120 and Leptin receptors). Interestingly, VitD3 given to mice fed HFD was unable to reverse the effect of HFD, led to increase tumour masses and reduction in $CD4^{+}CD25^{+}FOXP3^{+}$Tregs population. In conclusion, an obesogenic diet modulates melanoma tumour growth by establishing both chronic inflammation and potentially an immune suppressive microenvironment. Unexpectedly, vitamin D3 supplemented HFD promoted melanoma tumour progression in obese $LDLR^{-/-}$ mice, revealing the potential risk of vitamin D3 supplementation on a background of obesity mediated cancer.

Rafah Oday Al-Zubaidi

Statement of originality

This thesis submitted for the degree of PhD entitled "**Obesity and Vitamin D3 supplementation as modulators of tumour development and the immune microenvironment**" is based on work conducted by the author at the University of Leicester (England, UK) mainly during the period between October 2015 and September 2019. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University. This thesis results from work undertaken at the University of Leicester during the period of registration.

Dedication

To Raya and Zaydoon the most

Beautiful flowers in my life

My mother and father

With all respect

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Rafah Al-Zubaidi, Lee Machado and Cordula Stover. ***LDLR*$^{-/-}$ mouse melanoma model cautions against consumption of Vitamin D**. College of Life Sciences Poster Fair University of Leicester, Leicester, UK, March 2019. Appendix III.

Rafah Al-Zubaidi, Lee Machado and Cordula Stover. **The role of high fat diet (HFD) in melanoma tumour growth**. Images of Research competition.

LIST OF ABBREVIATIONS

BM	Bone marrow
bp	Base pairs
BSA	Bovine serum albumin
CD 4,25, 8	Cluster of Differentiation 4,25, 8
CD	Control Diet
cDNA	Complementary DNA
DAMP	Danger Associated Molecular Patterns
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylene diaminetetraacetic acid
ELISA assay	Enzyme-Linked immunosorbent assay
FC	Flow cytometry
FCS	Fetal calf serum
FAs	Fatty acids
FOXP3	forkhead fox P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte/Macrophage Colony-Stimulating Factor
HFD	High Fat Diet
HRP	horseradish peroxidase

IFN-γ	Interferon gamma
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IMS	Industrial Methylated Spirit
kb	Kilo bases
kDa	Kilo Dalton
LDLR$^{-/-}$	Low Density Lipoprotein Receptor knockout
LPS	Lipopolysaccharide
MCP-1	Monocytes chemoattractant protein
MDSC	Myeloid derived suppressor cell
mRNA	Messenger RNA
MTT	Methythiazolyldiphenyl- tetrazolium bromide
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified free fatty acid
nH2O	nanopure water
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
q PCR	Quantitative Polymerase Chain Reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

rpm	Revolutions per minute
RT	Room Temperature
S.C	Subcutaneous Injection
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
TAM	Tumour associated macrophages
TBS	Tris buffered saline
TEMED	N, N, N', N'-tetramethyl ethylene diamine
TLR-4	Toll-like Receptor 4
TME	Tumour microenvironment
TNF-α	Tumour Necrosis Factor Alpha
Treg cells	T- regulatory cells
VitD3	Vitamin D3
VLDL	very low density lipoprotein

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Chapter 1

General Introduction

General Introduction

1.1 Overview

In the past few decades, the world has seen a dramatic increase in individuals who are overweight or obese and these levels have reached epidemic proportions worldwide (Cao *et al.*, 2017). Several factors have been attributed to these increased rates including biological, genetic, environmental, cultural and psychosocial factors (Seiler *et al.*, 2018). According to the National Institutes of Health (NIH), obesity is a major health risk factor for several illnesses, including cardiovascular disease, type 2 diabetes, liver disease, kidney failure and different types of cancer, affecting both the risk of progression of disease and potential death (NIH, 2017). The expansion of obesity associated cancers emphasise a putative direct mutagenic effect of dietary components on tumour growth and progression (Stone *et al.*, 2018). It has been estimated that 14% of cancer deaths in men and 20% in women are attributable to excess body weight (Kolb *et al.*, 2016). However, these data are probably underestimated, as average weight has continued to increase in the global population over the same time.

1.2 Obesity

Obesity is the accumulation of fat in adipose tissue as a result of energy imbalance between overconsumption of calories within the diet, and the absence of compensatory energy expenditure (by exercise and everyday living) (Dalby *et al.*, 2018). Consumption of so-called ‘‘energy dense foods,’’ those high in fats and sugars, is significantly responsible for an increase in body weight gain in the population beside genetic susceptibility (Kheder *et al.*, 2017; Thorleifsson *et al.*, 2009). Obesity is determined by calculating the so-called body mass index (BMI; body weight [kg] /height [m^2]), People are considered obese if their BMI is higher than 30 kg/m^2 while, 18.5–24.9 kg/m^2 is within normal values and overweight as at intermediate values of 25–29.9 kg/m^2 and the term lean is referred to a body mass index below 18.5 kg/m^2 (Stone *et al.*, 2018).

In 2016, the World Health Organization (WHO) estimated that 39% of adults (39% of men and 40% of women) aged 18 years were overweight and 13% (11% of men and 15% of women) were obese (WHO, 2018a) (**Figure 1-1**). Furthermore, the percentage of overweight and obese people has risen dramatically among children and adolescents (aged 5-19) from just 4% in 1975 to just over 18% in 2016. The rise has occurred similarly among both boys and girls; in 2016, 18% of girls and 19% of boys were overweight. While just under 1% of children and adolescents aged 5-19 were obese in 1975, more 124 million children and adolescents (6% of girls and 8% of boys) were obese in 2016 (WHO, 2018a). In the UK the prevalence of overweight and obese adults (aged 16 and over) is between 59-65 % with (56-62% for females and 63-68% for males) (CRUK, 2018). According to the Eurostat **(**European statistics), the United States and Saudi Arabia represent the countries with the highest percentages of obese individuals while India has the lowest (Eurostat, 2016) (**Figure 1-2**).

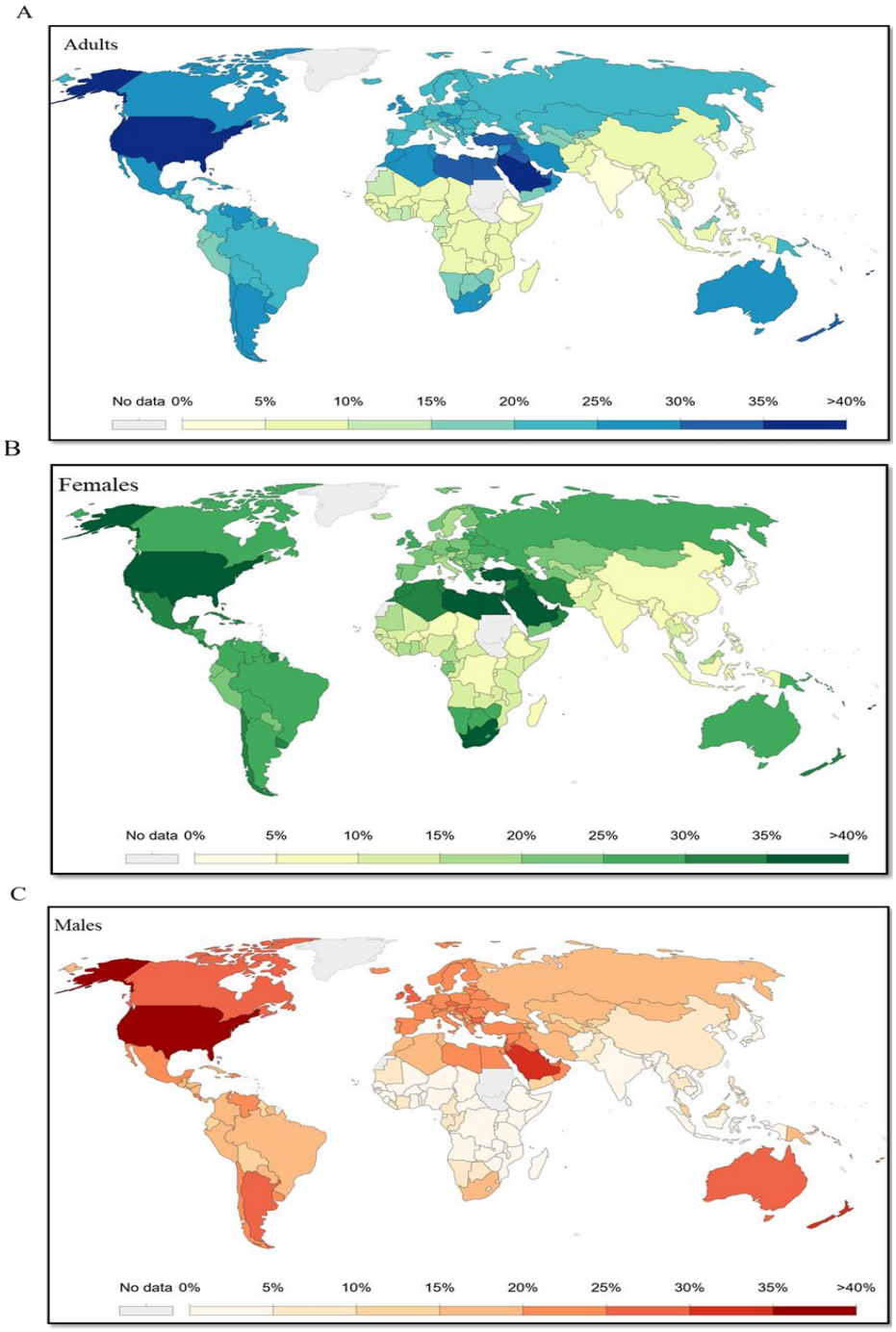


Figure 1-1: The global percentage of obese population.
(A) percentage of adults who are defined as obese according to their BMI >30 across the globe (**B**) The obese adult females (aged 18+ years) percentage while (**C**) percentage of obese adult males (aged 18 years and over) (WHO, 2018a).

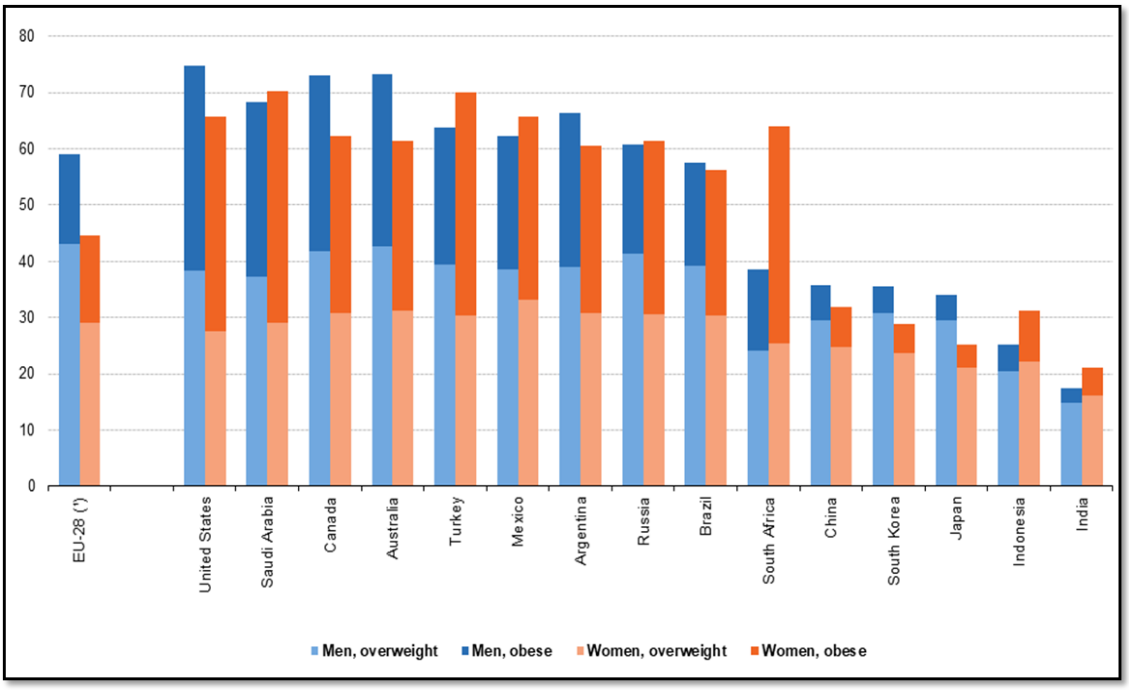


Figure 1-2: The prevalence of obesity and overweight in adults (aged 18 and over) in different countries (Eurostat, 2016).

1.3 Malignant melanoma

Malignant melanoma (MM) is a tumour that arises from transformed and genetically altered epidermal pigment-containing cells known as melanocytes in the skin as a result of complex interactions between genetic predisposition and environmental factors. Although, cutaneous melanoma is common form of melanoma, it can also arise in other regions such as mucosal surfaces and the uveal tract (Matthews *et al.*, 2017). While, it represents only 1% of all skin malignant tumours, cutaneous malignant melanoma is considered the most aggressive and highly metastatic form of skin cancer and is one of the leading causes of cancer-related mortality (Domingues *et al.*, 2018). However, in most cases melanoma is diagnosed at early stages as a contained primary tumour before metastases to other places (Dickson and Gershenwald, 2011).

At early stages the primary melanoma undergoes a 'radical growth' with less than 1 millimetre width. Then, tumour cells change their behaviour as they begin to move vertically into papillary dermal and epidermal tissue before spreading into the lymphatic system and distant organs to form malignant melanoma (MM) (Hershkovitz *et al.*, 2010). Therefore, and due to its distinctive pathological features (MM) can be divided in four histological types: Superficial spreading (SSM), nodular (NM), lentigo maligna

(LMM) and acral lentiginous melanomas (ALM) (Liu and Sheikh, 2014; Heistein and Acharya, 2018). Genetic alteration a common feature of neoplastic transformed cells, malignant melanoma typically exhibits a wide spectrum of DNA alteration (Bertolotto, 2013). Researchers found that the direct mutagenic effect of UV light on melanocyte DNA induces malignant changes in the skin layer. This effect stimulates production of growth factors that activate mutation of genes including BRAF, NRAS and NF1 (proto-oncogenes that encode proteins responsible for promoting cell growth and proliferation) (Leiter and Garbe, 2008). Another important effect of UV radiation is to inactivate tumour suppressor genes for example, cyclin dependent-kinase inhibitor 2A (CDKN2A) which prevents cells from rapidly proliferating (an uncontrolled division). Additionally, increased exposure to UV light also inhibits immune responses and, stimulates melanin pigment production which leads to increase intracellular stress and ROS production. These can subsequently cause DNA damage, mutation and suppresses apoptosis (Liu and Sheikh, 2014).

In addition, Leonardi *et al.* (2018), reported that genetic alterations and dysregulation of the nuclear factor (*NF)-κB* pathways mediate tumour microenvironmental alterations, characterized by overexpression of different proteins, particularly, the overexpression of matrix metalloproteinase (MMPs) such as MMP-9 and MMP-2. These proteins induce degradation of the components of the extracellular matrix, which facilitate tumour progression. An overall alteration in molecular pathways and genetic dysregulation in melanocytes lead to melanomas with diverse biology and the combination of intracellular signal transduction molecules coordinate cancer cell ability to invade and proliferate (Kato *et al.*, 2002; Palmieri *et al.*, 2012). More recently, Xia *et al.* (2017) found that diets rich in fat increase serum levels of ketone body acetoacetate leading to accelerate tumour growth potential of BRAF expressing human melanoma cells in xenograft mouse models. This indicates a possible pathogenic link between dietary components and specific oncogenic mutations that occur in cancers with distinct oncogenes requiring different metabolic alterations for full tumour progression.

1.3.1 Risk factors

Environmental factors, hereditary as well as acquired genetic mutations are considered as important factors in melanoma cancer aetiology (Falzone *et al.*, 2016; Hawkes *et al.*, 2016). Several studies showed that exposure to solar ultraviolet (UV) radiation are causative risks for melanomas cancer in approximately 65% of all melanoma causes (Williams and Ouhtit, 2005). UV rays can cause melanoma either by DNA damage which may induce mutation in the epidermal cells or by up-regulation of gene expression through intracellular signal transduction pathways which may contribute to the melanoma cancer (Lin and Fisher, 2007). Oncologists found that UV-induced DNA damage enhances keratinocytes to produce melanocyte stimulating hormone (MSH) which binds to melanocortin receptor1 (MC1R) on the melanocytes resulting in the release of melanin. This pigment ultimately provides a molecular shield against solar radiation, therefore preventing further DNA alterations (Leonardi *et al.*, 2018). Additionally, hereditary factors such skin type, hair colour, and family history is associated with an increased susceptibility to melanoma cancer risk. Furthermore, some familial mutations have been found to increase susceptibility to melanoma development. These families harbour mutations in the tumour suppressor gene CDKN2A which increases their risk of melanoma (Soura *et al.*, 2016).

Additionally, white people with red hair are more susceptible to developing melanoma cancer compared to dark skin individuals due to genetic variation in the melanocortin receptor-1 (MC1R) gene, which leads to the secretion of pheomelanin instead of melanin (Thompson *et al.*, 2005). Melanoma incidence also varies by sex, which is also associated with differences in the anatomical distribution of melanoma lesions. The mortality rate of melanoma is also different depending on region, ethnicity, age, and sex. It has been reported that the incidence of MM is more frequent in men and they exhibit a higher mortality rate than women (Matthews *et al.*, 2017).

1.3.2 Epidemiology of Malignant Melanoma

According to World Health Organisation (WHO) statistics, the worldwide incidence of melanoma has risen faster in the last 50 years with global estimated rate over 100,000 melanoma skin cancers annually (WHO, 2019). It has been reported that MM is among 20 lethal cancers in the global (**Figure 1-3**) (CR, 2019), and it is the fifth most common cancer in the UK (CRUK, 2018). Over the last few decades, the incidence and mortality rates of MM in the UK have dramatically increased for both genders with around 15,400 new cases every year. Between 1993 and 2015, the incidence rates have significantly increased 10 to 28 fold in males and 13 to 24 in females, respectively (**Figure 1-4**) (Misko, 2019). Several studies reported an association between melanoma incidences and Geographical location (Chang *et al.*, 2009).

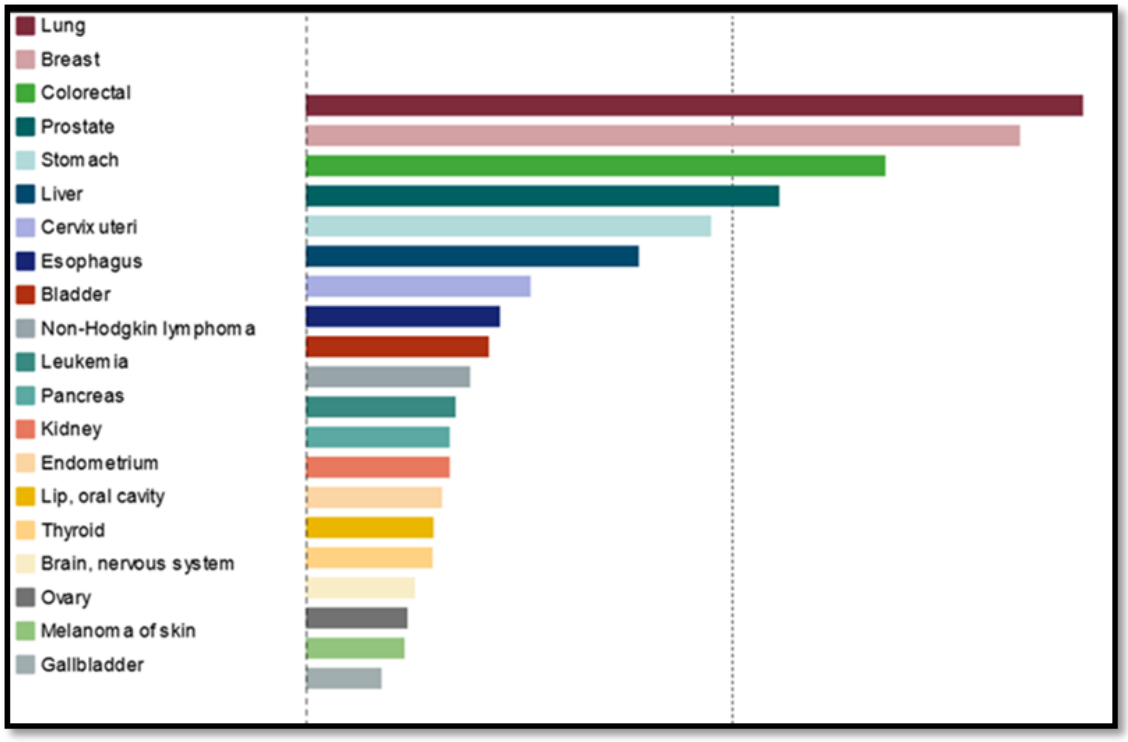


Figure 1-3: The incidence of cancers in the world wide in 2012 (CR, 2019).

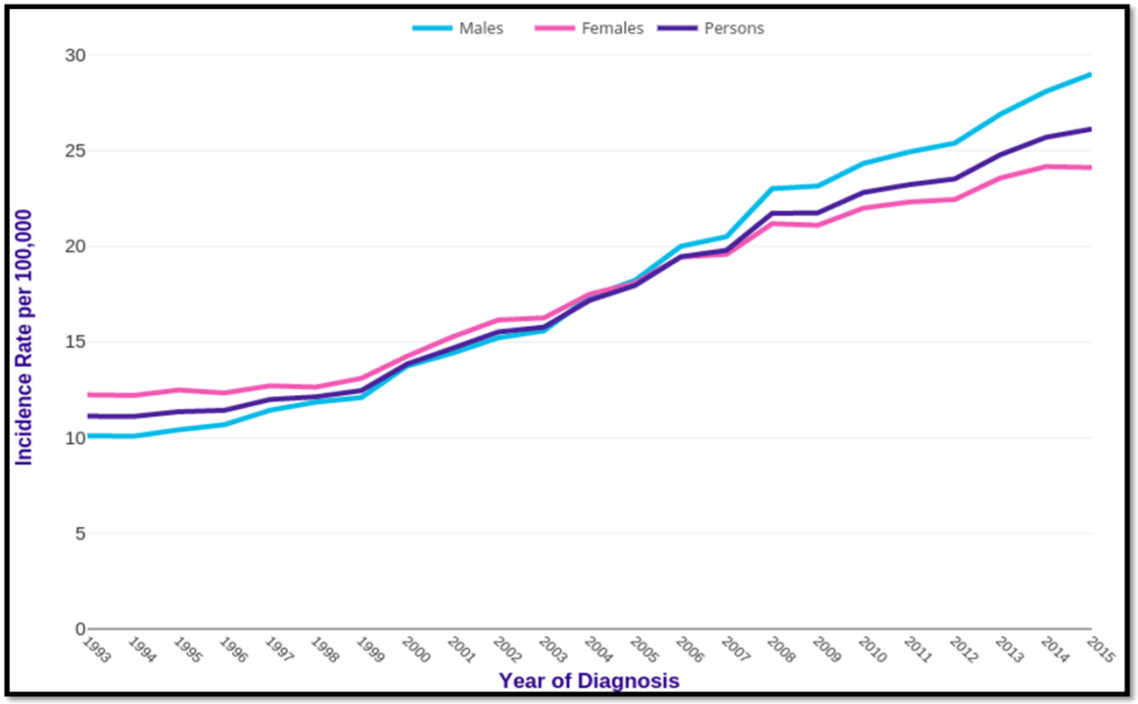


Figure 1-4: Incidence rate of malignant melanoma (MM) in both sexes in the UK from 1993-2015 (Misko, 2019).

1.3.3 Obesity and cancer morbidity and mortality

Being overweight or obese has long been understood to increase the risk of cancer and cancer related mortality (Akinyemiju *et al.*, 2018), and is considered causal factor in various cancer such as stomach, bowel, breast, kidney, liver, uterus oesophageal, pancreas, gall bladder and thyroid (Stone *et al.*, 2018). According to the world health organization (WHO), nearly one third of deaths from cancer are attributed to increase in body mass index, low consumptions of fruit and vegetable, lack of physical activity, tobacco use, and alcohol consumption (WHO, 2018b). Data published over the last 25 years emphasize that obesity is associated with high cancer mortality rates and may account for 14% and 20% of cancer related deaths in overweight men and women under 50 years of age, respectively. Furthermore, it has been estimated that nearly 52% and 62% of the cancer related death occurred in morbidly obese (BMI > 40 kg/m2) men and women, respectively (Deng *et al.*, 2016). A study in 2018 reported that a high body mass index greater than 25 kg/m^2 and diabetes are risk factors for 5.7% of worldwide cancer burden in 2012, and (24.5%) cases of liver cancer in men and (38.4%) cases of breast cancer in women were attributable to these factors. Individually, an increase in BMI is responsible for nearly twice as many cancer cases as diabetes. Furthermore,

diabetes and high BMI may be responsible for (41.9%) of liver cancer and (23.1%) of colorectal tumour in men, while breast and endometria cancers are among the highest cancers related to overweigh or obesity in women (29.4% and 24.3%) respectively (Pearson-Stuttard *et al.*, 2018). Published data estimate that overweight and obesity causes 6% of all cancer cases in the UK (CRUK, 2018), while others have claimed that the relative risk of mortality from cancer, attributable to obesity is 14.2% in men and 19.8% in women (Stone *et al.*, 2018).

1.4 Low-density lipoprotein receptor-deficient (*LDLR*$^{-/-}$) mice as a model system

The metabolic syndrome (MetS) is usually characterized by obesity associated with other metabolic abnormalities such as hypertriglyceridemia, low levels of high density, lipoprotein, high blood pressure and elevated fasting glucose levels (Wong *et al.*, 2016). Obesity is on the rise worldwide, and its association with these metabolic symptoms increases the risk for different diseases and many types of cancer, therefore it has become imperative to develop animal models of MetS in order to identify the mechanisms behind this action (Kennedy *et al.*, 2010). The low density lipoprotein (LDL) receptor *(LDLR*) is expressed by many types of cells especially by the liver and it is responsible for removing LDL cholesterol from the blood in a process known as endocytosis (Barrett and Watts, 2002). This receptor has the ability to bind to LDL with high affinity and cycles many times to deliver large amounts of cholesterol to body tissue (Nakaya *et al.*, 2009), thereby maintaining normal levels of LDL in the circulation (Schreyer *et al.*, 2002). The deficiency in this receptor increases circulating fatty acids in blood (Khan *et al.*, 2006; de Ferranti and Mozaffarian, 2008). The accumulation of fatty acid can lead to inflammation and apoptosis through oxidative or endoplasmic reticulum stress which in turn enhances cytokine production such as TNFα and IL-6 and thereby generates ROS in the tissues (Yu *et al.*, 2002; Willecke *et al.*, 2015).

Low-density lipoprotein receptor deficiency is one of the most severe forms of human hyperlipidemia, familial hypercholesterolemia, occurred due to mutations in the low-density lipoprotein receptor (*LDLR*) which induces increased in the levels of the atherogenic lipoprotein LDL (Kennedy *et al.*, 2010). The level of plasma LDL

cholesterol was increased in *LDLR* deficient mice fed a diet rich in fat and associated with the development of atherosclerotic lesions (Ishibashi *et al.*, 1994). These mice developed moderate hypercholesterolemia on normal chow diet with lipoprotein profiles similar to humans. On a high-fat diet containing 21% fat, *LDLR*$^{-/-}$ mice develop severe hyperlipidemia and extensive atherosclerosis (Ishibashi *et al.*, 1994; Coenen *et al.*, 2007). Moreover, it has been shown that *LDLR*$^{-/-}$ mice are more susceptible to gain weight and increase in hypertriglyceridemia compared to C57BL/6 wild type mice when fed on high fat diet (Schreyer *et al.*, 2002). In fact, when *LDLR*$^{-/-}$mice are given a diet with greater than 20% fat content they also become obese and display insulin resistance (IR) (Wu *et al.*, 2006). Therefore, the *LDLR*$^{-/-}$ mouse model can be particularly useful when studying diet-induced obesity and IR in the presence of hyperlipidaemia (**Figure1-5**).

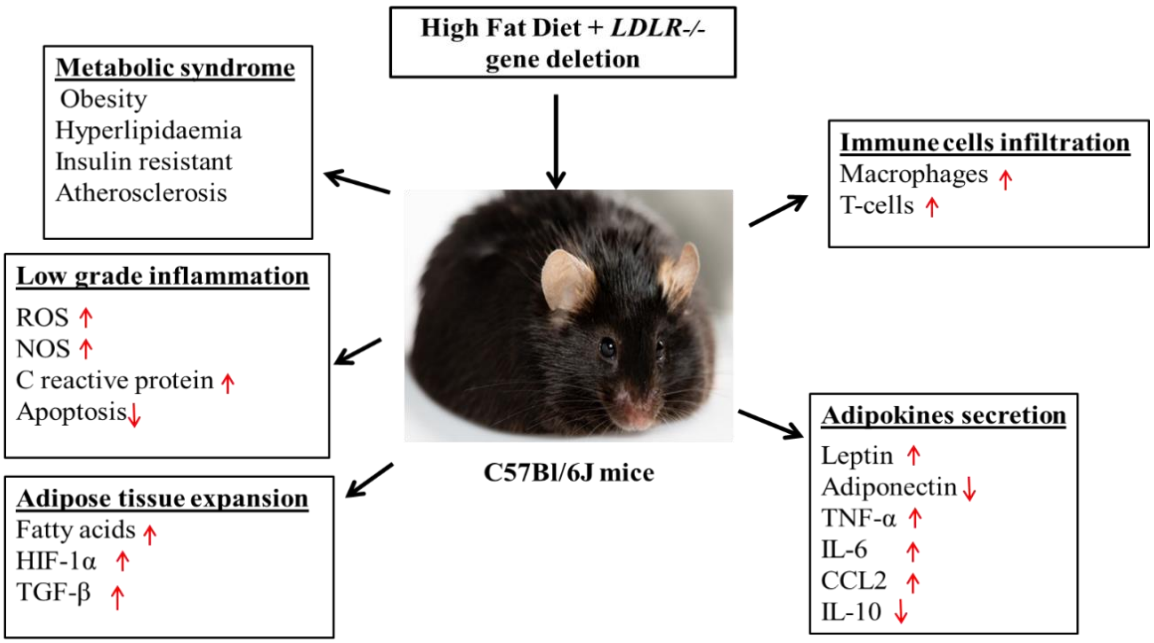


Figure1-5: Diet-induced obesity in *LDLR*$^{-/-}$ mouse model and suggested contributory factors.

1.5 The Tumour Microenvironment (TME)

The tumour microenvironment (TME) hosts a complex network of heterogeneous stromal cells such as cancer associated fibroblasts, endothelial cells, infiltrating immune cells, adipose cells, extracellular matrix (ECM) and a lymphatic vascular network with either overlapping or opposing functions based on the dominant signals within this milieu (Ramamonjisoa and Ackerstaff, 2017). Infiltrating leukocyte likes lymphocytes and myeloid cells from early stages represent the central cellular content of solid tumours. Due to their function and number, myeloid cells represent a prominent population among these leukocytes, supporting tumour progression and even having prognostic value. Tumour-associated myeloid cells (TAMC) can be divided into five distinct populations: Tumour-associated macrophages (TAM),Myeloid-derived suppressor cells (MDSC), Tumour-associated neutrophils (TAN),Tumour-associated dendritic cells (TADC) and Monocytes expressing the angiopoietin-2 (Ang-2) receptor Tie2 known as Tie2-expressing monocytes or (TEM) (Singh *et al.*, 2017). Intercellular communication is responsible for proliferation and invasion of the tumours by secreting growth factors, chemokines and matrix-degrading enzymes (Belli *et al.*, 2018).

Deviated from their normal physiological roles, immune, vascular, and stromal cells within the tumour microenvironment are often dysfunctional and are used by cancer cells to support tumour development and progression. Moreover, oncologists reported that the biochemical and biophysical features of the tumour microenvironment (TME) are distinct from that in normal tissues and the composition of the stroma in the TME are also varied between tumours (Yang, 2017). The importance of understanding the structure of the TME comes from their influential ability in modulating cancer therapeutic responses and their role in multiple stages of cancer progression (Cairns *et al.*, 2006). Accumulated evidence indicates that interaction between cancer cells and infiltrating immune cells contributes to the maintenance of a chronically inflamed TME with pro-tumorigenic immune phenotypes which facilitate tumour growth and development (Seager *et al.*, 2017). These immune cells are of haematopoietic origin and arise in the bone marrow and they can be either progress through the lymphoid lineage (such as T, B lymphocytes and natural killer cell) or down the myeloid lineage (including macrophages, neutrophils and myeloid-derived suppressor cells (MDSC) (Pattabiraman and Weinberg, 2014). Tumour promoting inflammation and avoiding immune destruction have been recognised as two cancer hallmarks and studies have

shown that immune cells may exhibit immunosuppression action to achieve this (Wang *et al.*, 2017). Due to their important contribution in pathogenesis of various types of cancer, T-regulatory lymphocytes (Tregs), macrophages and adipocytes will be explored in detail in the following section.

1.5.1 Regulatory T cells (T-regs)

Regulatory T cells (T-regs) are a diverse immunosuppressive subset of $CD4^+$ T lymphocytes that play essential roles in maintaining immune homeostasis and self-tolerance (Wang *et al.*, 2018).These cells are also involved in controlling autoimmunity, infection, inflammation, and tumour immune responses (Chaudhary and Elkord, 2016). Regulatory T cells can be identified by cell surface markers ($CD4^+$ CD25 high) and the transcription factor forkhead box P3 (FOXP3). However, CD25 and CD4 are also expressed by other T cells (Sharma *et al.*, 2006). For example, CD25 is a high-affinity receptor for interleukin-2 (IL-2) and is also expressed on Natural killer (NK) cells and Cytotoxic T Lymphocytes (CTLs) and play influential roles in their proliferation (Borrelli *et al.*, 2018). Therefore, (FOXP3) is considered as a specific marker and is a master regulatory transcription factor for T-regs (Najafi *et al.*, 2019). T-regs can be found as thymic T-regs cells also known as a natural T-regs cells (nT-regs) where they have developed in the thymus by the stimulation of self-antigen or induced T-regs cells (iT-regs) which are differentiated in peripheral tissues upon antigen and cytokine stimulation (Deng, 2018).

In healthy conditions, T-regs represent about 4–5% of the peripheral $CD4^+$ T-cell pool (Mougiakakos *et al.*, 2010). In contrast, the number of these was significantly increased within the tumour microenvironment and is negatively associated with poor survival rates in cancer patients (Fu *et al.*, 2018). Murine experiments have shown that T-regs cells are markedly increased in tumour bearing mice and the depletion or inhibition of these cells can potentially enhance anti-tumour responses (Ma *et al*., 2007). Importantly, Tregs can suppress the immune responses in different ways such as suppression of tumour specific $CD8^+$ cytotoxic T cells, $CD4^+$ T cells, NK cells and induction of immunosuppressive cytokines (TGF-β and IL-10). The overexpression of CD25 molecules attenuate anti-tumour cytokines such as IL-2, IL-7, and IL-12 and finally expression of inhibitory molecules including Cytotoxic T Lymphocytes Antigen-4 (CTLA-4) and programme cell death (PD-1) inhibit the interaction of antigen presenting

cell APCs with cytotoxic T cells. (Baumgartner *et al.*, 2007). In murine models of melanoma, T-regs depletion induced anti-tumour immunity and improved tumour clearance and survival (Jacobs *et al.*, 2012). Accumulating evidence demonstrates increases in T-regs percentage in peripheral blood of patients with metastatic melanoma (Mougiakakos *et al.*, 2010). Furthermore, research showed that T-regs in melanoma patients are functionally suppressive and they are highly enriched in the local tumour microenvironment (Jandus *et al.*, 2008; Ladanyi *et al.*, 2010). In addition to that, secretion of local immunosuppressive factors such as TGF-β promoted T-reg survival and proliferation in melanoma tumours (Nizar *et al.*, 2010). Interestingly, pro-inflammatory cytokines TNF-α induce up-regulation in proliferation, survival, stability, expression of $CD25^+$, $Foxp3^+$, as well as differentiation and suppressive function of murine T-regs. Similarly, the same authors reported that TNF- α doesn't impair the suppressive activity of human T-regs (Salomon *et al.*, 2018). Several mechanisms may contribute to the accumulation of T-regs in the tumour microenvironment. It has been postulated that T-regs migration and aggregation could be controlled by local chemokine induction (Ouyang *et al.*, 2016). In human ovarian carcinomas, T-reg cells migration was enhanced by tumour cells secreted CCL22 (Curiel *et al.*, 2004). This is important because CCL22 could control CCR4-expressing T-regs migration in other tumours such as breast and prostate (Miller *et al.*, 2006; Wei *et al.*, 2006). However, the migration of T-regs (expressing-CCR4) could be enhanced by CCL2 instead of CCL22 in murine melanoma (Kimpfler *et al.*, 2009). Furthermore, in a murine model of pancreatic cancer, disruption of CCR5/CCL5 signalling impaired intra-tumoral T-regs accumulation and regression of established tumours (Tan *et al.*, 2009).

This also applied to the chemokine receptor CXCR3, which was found to induce accumulation of Intra-tumoural T-regs expressing $CXCR3^+$ in human ovarian, colorectal, and hepatocellular carcinomas (Redjimi *et al.*, 2012; Yang *et al.*, 2011). It has been shown that $CCR10^+$ T-regs are recruited during hypoxia by responding to CCL28 which was enhanced during tumour progression. The result was secretion of vascular endothelial growth factor A (VEGF-A) and subsequent promotion of tumour angiogenesis of mouse ovarian cancers (Facciabene *et al.*, 2011). In the tumours of B16 melanomas, CCL21/CCR7 signalling induced recruitment and accumulation of T-regs which accelerated tumour progression (Shields *et al.*, 2010). Recently, significant advances have been made in furthering our understanding of the actions of specific

dietary components on the immune system. Among the different proposed channels through which dietary components affect immune responses, regulatory T-cells (T-regs) are emerging as main targets for the dietary prevention of chronic inflammatory diseases (Issazadeh-Navikas *et al.*, 2012). Environmental factors and certain dietary component may play an influential role in T-regs differentiation (Kim and Lee, 2013). Importantly, adipokines such as leptin and Adiponectin secreted by adipocytes which regulate food intake and metabolism at the hypothalamic level can also affect the generation and proliferation of T-regs (Galgani *et al.*, 2016). HFD fed mice caused a gradual decrease in hepatic T-regs. However, splenic T-regs numbers were not affected by this diet (Issazadeh-Navikas *et al.*, 2012).

1.5.2 Macrophages

Macrophages (MΦs) are the basic component of the innate immune system that play a broad role in the maintenance of tissue homeostasis and stress responses (Gordon and Pluddemann, 2017). They are part of the mononuclear phagocytic system (MPS) which protect the host against any pathogenic infections (Liu and Cao, 2015). These cells originate in the bone marrow from hematopoietic stem cells and upon tissue damage or infection; monocytes are rapidly recruited and differentiate into tissue macrophages (Kratofil *et al.*, 2017). Macrophages acquired distinct phenotype and functional activities directed by the local tissue and immunological microenvironment (Panni *et al.*, 2013). The main feature of macrophages is their subset heterogeneity and plasticity enabling them to change their phenotypic behaviour in response to innate and adaptive immune signals (Gordon and Taylor, 2005). Conventionally, Macrophage responses can be divided into two major subtypes; classically activated/ anti-tumour M1 like- cells (M1-MΦs), and alternatively activated /pro-tumour macrophages expressing an M2 like- phenotype (M2-MΦs) (Yahaya *et al.*, 2019). M1-macrophage responses are driven by Toll-like receptor (TLR) agonists, cell-mediated immune responses including secretion of IFN-γ, TNF-α and GM-CSF and bacterial moieties such as lipopolysaccharide (LPS). They express pro-inflammatory cytokines such as IL-6, IL-12 and nitric oxide (NO) or produce reactive oxygen intermediates (ROI) to protect against pathogenic infection and kill tumour cells (Singh *et al.*, 2017).

Conversely, macrophage M2-polarization is driven by Th_2 cytokines, such as interleukin (IL)–4, IL-10, and IL-13 and they exert anti-inflammatory and pro-tumorigenic activities (Hagemann *et al.*, 2009). Importantly, macrophages also differ in their ability to utilize Arginine (amino acid), M2-MΦs metabolize arginine to generate ornithine and urea through the arginase pathway which is used for cell proliferation and collagen biosynthesis (tissue repair). While, M1-MΦs used Arginine to produce large amounts of toxic NO (as part of the M1 killing machinery) by inducing enzyme inducible NO synthase (iNOS) (Ley, 2017). Within the tumour, macrophages are a major stromal component in the tumour- microenvironment (TME), and they constitute up to 50% of a tumour mass, where they are commonly referred to as Tumour-associated macrophages (TAMΦ) (Sica and Mantovani, 2012, Kim and Bae, 2016). Several studies have indicated that TME educated macrophages to exhibit alternatively activated M2-MΦs (Kim and Bae, 2016). The high frequencies of these is largely associated with poor prognosis in most malignant tumours (Zhang *et al.*, 2012). TAMs contribute to tumour progression by facilitating angiogenesis, promoting tumour invasiveness, and inhibiting protective adaptive immunity and mediating cancer-related inflammation. They can also influence tumour relapse after anticancer therapies (Poh and Ernst, 2018). TAM promote angiogenesis by increasing the production of mediators such as VEGF, cyclo-oxygenase-2 (COX2)–derived prostaglandin E2, IL-10 and IL-8 (Van Ginderachter *et al.*, 2006). Furthermore, tumour associated macrophages (TAM) mediate immunosuppression action by increasing expression of CCL17, CCL22 and CCL24 which facilitate recruitment of Tregs into the TME (Panni *et al.*, 2013). TAMs also express programme cell death ligand (PDL-1) and PDL-2, which can inactivate T-cells. Additionally, they have the ability to down-regulate their expression of major histocompatibility complex class II (MHC II). Thus, TAMs suppress the antigen presentation and T-cell responses in the tumour (Hamilton, 2008). Another feature of TAMs is their ability to sustain inflammation in the TME by deactivating NF-κB in response to signals such as LPS and TNF-α which promote pro-tumour phenotypes (Mantovani *et al.*, 2008). During melanoma development, activation of macrophages leads to the production of TGF-β, TNF-α, IL-1α and extracellular proteases. Accordingly, melanocytes respond by expressing IL-8 and vascular endothelial growth factor (VEGF)-A, thereby inducing vascular angiogenesis (Torisu *et al.*, 2000).

In murine TME, TAMs down-regulate IL-12 and increase express of specific genes such as arginase-1(Arg-1) and macrophage galactose –type C-type lectin–2(Mgl2) (Mantovani *et al.*, 2002). However, the expression of inducible nitric oxide (iNOS or NOS2, an enzyme expressed by M1 macrophages) is also demonstrated in these cells (Kusmartsev and Gabrilovich, 2005). In murine melanoma tumour, repolarization of TAMs phenotype from M2- MΦs into M1-MΦs by immunostimulatory agents led to regression in tumour growth (Jarosz-Biej *et al.*, 2018).

1.5.3 Adipocytes

Adipocytes, the basic cellular component of adipose tissues, have an important role in metabolism, inflammation, and cancer (Desruisseaux *et al.*, 2007). In spite of being traditionally considered as fat-storage depots in the form of triglycerides, it has become clear that adipocytes have a significant role in regulation of whole body energy homeostasis due to their secretion of wide range of cytokines, hormones and growth factors, collectively referred as adipokines (Cinti, 2012). Accumulating evidence showed that within the tumour microenvironment (TME), adipocytes also known as tumour-associated adipocytes (TAAs), have an essential role in growth and progression of malignant tumours by providing fatty acids, adipokines and proteases (Catalan *et al.*, 2013). Interestingly, tumour cells utilise fatty acids-derived adipocyte for energy production, therefore, sufficient amounts of fatty acids/lipids from tumour-associated adipocytes favour uncontrolled growth and development of malignancy (Ray and Cleary, 2017). The effect of fat cells on growth and survival of cancer cells has been demonstrated in various cancer cells (Diedrich *et al.*, 2015).

The interaction between cancer cells and adipocytes within the TME induced lipolysis in adipocytes which enable FAs to transfer from adipocytes into tumour cells, leading to β–oxidation in tumour cells (Yang *et al.*, 2018). Culturing ovarian cancer cells with adipocytes enhanced the transfer of lipids between cells, inducing fat cell-driven lipolysis and up-regulation of β-oxidation in cancer cells, supporting their rapid division (Nieman *et al.*, 2011). Notably, β-oxidation has also been considered as an important source of energy in various cancers such as prostate cancer (Gazi *et al.*, 2007), and breast cancer (Wang *et al* .2017). Similarly, due to their anatomical location, adipocytes supply melanoma cells with high levels of fatty acids, fuelling proliferation and invasion which supports their growth and progression (Zhang *et al.*, 2018).

1.6 Adipocytokines (Adipokines)

Adipokines are defined as group of pharmacologically active, low molecular weight proteins secreted by adipose tissue that acts as endocrine and paracrine hormones (Bluher, 2014). The importance of these peptides comes from their ability to work as a network to modulate appetite and satiety, glucose and lipid metabolism, inflammation and immune function locally and systemically (Al-Suhaimi and Shehzad, 2013). Excessive secretion of inflammatory adipokines by adipose tissue is the main feature of obesity related inflammation (Ouchi *et al.*, 2011).

These adipokines have several mediators and include Leptin, Adiponectin, Tumour Necrosis Factor alpha (TNF-α), interleukin 6 (IL-6), Resistin, interleukin1 (IL-1) and monocyte chemotactic protein-1(MCP-1) (Fisman and Tenenbaum, 2014). In spite of the pro-inflammatory action for the majority of adipokines, Adiponectin conversely has anti-inflammatory and anti-tumour action (Divella *et al.*, 2016). In addition to their role in regulating lipid metabolism and glucose homeostasis, adipokines are also associated with multiple pathologies related to excess fat mass including cancer development (Catalan *et al.*, 2013). Several studies have found an association between circulating adipocyte-derived hormones and increased prevalence of malignancies (Housa *et al.*, 2006). The paracrine loop between adipocytes and immune cells is altered in obesity, due to a significant increase in adipokines production which leads to dysregulation in immune cell cytokine (Booth *et al.*, 2015). Emerging evidence shows that obesity-induced adipokines secretion promote dysregulation in mitochondrial activity which induces inhibition of the tumour suppressor protein p53 in normal tissue, thereby driving tumorigenesis (Diedrich *et al.*, 2015). Therefore, the possible role of these adipokines in the pathogenesis of malignant tumours will be reviewed in details.

1.6.1 Tumour necrosis factor (TNF-α)

TNF-α, a pro-inflammatory adipokine that may contribute to the pathogenesis of obesity and, it is positively correlated with insulin resistance (Jung and Choi 2014). TNF superfamily members have an influential role in regulating various cellular and biological functions such as cell differentiation, proliferation, apoptosis and energy metabolism (Cawthorn and Sethi, 2008). Adipocytes and infiltrated macrophages express an increased level of TNF-α during HDF–induced obesity (Weisberg *et al.*,

2003). Interesting, TNFα production in macrophages was increasingly enhanced by free fatty acid (FFA) during obesity and in turn, TNFα induced lipolysis to increase fatty acid release from adipocyte (Nguyen *et al.*, 2005). Obese animals showed improvement in insulin sensitivity and inflammatory status after deletion of TNF-α or its receptors (Wascher *et al.*, 2011). It has been suggested that TNF-α inhibits insulin receptor signalling pathways thereby promoting insulin resistance (Shi *et al.*, 2014).

Adipose-derived TNF-α could be a major mechanistic link between obesity and cancer related inflammation (Cao, 2014). Additionally, an association was found between elevated circulating levels of TNF-α and progression and development of different cancers (Catalan *et al.*, 2013). However, this multi- functional cytokine was firstly identified as a cytokine with anti-tumour properties but later the active role of TNF-α in cancer pathogenesis was considered (Trayhurn and Wood, 2004). TNF-α is involved in cancer at all stages of tumorigenesis which include, tumour cell transformation, survival, proliferation, invasion, angiogenesis and metastasis (Divella *et al.*, 2016). This cytokine regulates apoptosis through binding to its receptor (TNF-R1), downstream signalling cascade and thereby activate nuclear factor (NF) –κB. This activation induces up-regulation of several negative regulators of apoptosis, such as c-FLIP and cIAP1, which thereby promote cell survival (Van Kruijsdijk *et al.*, 2009). Furthermore, TNF-α may enhance tumour progression by stimulating a constitutive network of factors, including VEGF and the chemokines CXCR4 and CXCL12 (Kulbe *et al.*, 2007). Importantly, systemic levels of TNF-α might also be associated with the early development of some cancers, as a study showed that elevated TNF-α levels were correlated with increased risk of colorectal adenomas (Kim *et al.*, 2008). Moreover, TNF-α can regulate other adipokines which enhance oncogenesis. *In vitro* study showed down-regulation in Adiponectin mRNA expression within adipose tissue in response to TNF-α, an action that may promote cancer development (Bruun *et al.*, 2003). Moreover, TNFα might enhance leptin action by up-regulating leptin receptor expression (Gan *et al.*, 2012). However, like any other factors implicated in cancer pathogenesis, the precise action of TNF-α may vary according to type, stage of cancer and local microenvironment (Stone *et al.*, 2018).

1.6.2 Interleukin-6 (IL-6)

IL-6 is an adipokine produced by adipose tissue and belongs to a group of pro-inflammatory cytokines, whose expression levels increase in obese mice and subjects (Vainer *et al.,* 2018). IL-6 plays an important role in maintenance of whole body glucose metabolism and metabolic homeostasis (Roh *et al.*, 2016). Obese mice showed improvement in hepatic insulin action after depletion of IL-6 by neutralizing antibodies. Furthermore, growing evidence continues to indicate the role of several pro-inflammatory cytokines in regulating cancer cell growth and their contribution in cancer pathogenesis. Amongst these, IL-6 seems to take a centre stage in a number of malignant conditions, including both haematopoietic and solid tumours (Waldner *et al.*, 2012). IL-6 a pleiotropic cytokine, whose release is associated with insulin resistance, immune responses as well as inflammation-associated carcinogenesis and metastatic potential (Diedrich *et al.,* 2015). The expression of IL-6 is positively associated with tumour promotion and progression in several tumours. However, this may depend on tumour types (Eikawa *et al.,* 2010). IL-6 mediates its action by binding to either cell surface receptor (mIL-6R) called the classical pathway, or by binding to the soluble receptor (sIL-6R) in the tissue through the trans-signalling pathway. Furthermore, IL-6 regulates the expression of genes involved in the different steps of tumour growth by action of the Janus kinase-2 (JAK2) /signal transducer and activator of transcription-3 (STAT3) signalling pathway (Cao, 2014). This is an important anti-apoptotic and proliferative mechanism in tumours, through direct activation of IL-6 (Wang *et al.*, 2013a; Loffler *et al.*, 2007). IL-6 also inhibits the differentiation of dendritic cells, enhances immune tolerance and impairs T cell immune-surveillance and reduces cytotoxicity (Stone *et al.*, 2018). Additionally, the secretion of IL-6 into the circulation is promoted by Insulin and enhances TNF- α gene expression within adipose tissue, which provides intriguing links between the occurrence of inflammation and insulin (Krogh-Madsen *et al.*, 2004). Intriguingly, anti-IL-6 antibody treatment attenuates tumour growth in obese mice fed on a high fat diet but not on mice fed on a normal diet, suggesting that a pro-inflammatory microenvironment in an obese status could stimulate tumour growth (Duong *et al.*, 2017).

1.6.3 Interleukin – 10 (IL-10)

Interleukin 10 (IL-10) is an anti-inflammatory cytokine that plays a critical role in down-regulating inflammatory and autoimmune pathologies (Ip *et al.*, 2017). IL-10 was initially described as a T helper 2 (TH2) cytokine that modulates the activity and/or differentiation of innate immune cells, keratinocytes, endothelial cells and suppresses the activation and effector functions of T cells (Jung *et al.*, 2004). T helper cells, monocytes, macrophages and dendritic cells are considered to be main producers for IL-10, however B cells, cytotoxic T cells, NK cells, mast cells, and granulocytes like neutrophils and eosinophils are also capable of producing this cytokine (Hong *et al.*, 2009). The immunosuppressive activity of this cytokines is mediated by heterodimeric IL-10 receptor (IL-10R1, IL-10R2) (Iyer and Cheng, 2012). Binding of IL-10 with it is receptors leads to activate JAK/STAT signalling pathways which induce large changes in the expression profile of immune-modulatory genes which decrease antigen presentation and phagocytosis, and concomitantly enhance the inhibitory, tolerance, and scavenger functions of these cells (Wills-Karp *et al.*, 2010). The main actions of this pleiotropic cytokine is its ability to suppress the production of pro-inflammatory cytokines such as TNF-α , IL-1 and IL-12, which are synthesized by macrophages when a so-called M1 skewed response is required, which leads to decrease interferon-γ (IFN-γ) production by macrophages and Th1 lymphocytes and inhibit cell-mediated immune responses, while concomitantly enhancing humoral immunity. Furthermore, IL-10 has been shown to prevent a number of immune functions, i.e. T-lymphocyte proliferation, Th1-type cytokine production, and lymphokine-activated killer cell cytotoxicity. Additionally, IL-10 inhibits antigen-specific T-cell proliferation by reducing the antigen-presenting capacity of monocytes through down-regulation of histocompatibility complex class II (MHC-II) expression. Since IL-10 has immunosuppressive and anti-inflammatory characteristics and is produced by some types of cancer, it has been speculated that its production by cancer cells may contribute to the escape from immune surveillance (De Vita *et al.*, 2000). IL-10 mRNA is expressed in tumour tissues of ovarian, breast, renal, lung origin, and skin cancer (Sato *et al.*, 2011). IL-10 increases the expression of co-inhibitory molecules such as B7-H1 within the melanoma tumour microenvironment (TME) (Fujimura *et al.*, 2012). In the obese context, animal experiments found that HFD-induce obesity decrease the serum

levels of IL-10, and this reduction plays a crucial role in terms of obesity–induced inflammation (Kondo *et al.*, 2018).

1.6.4 Leptin and Leptin receptors (ObRs)

Leptin is one of the most influential adipokines in metabolic regulation, regulates weight gain through signalling nutritional status to other organs (mainly to the hypothalamus), and results in the production of molecules that modulate food intake and energy expenditure (Perry and Wang, 2012). Humans and rodents, plasma leptin levels are highly correlated with body fat (Greenberg and Obin, 2006). It has been suggested that leptin which is a multifunctional hormone, secreted primarily from adipocytes and elevated during obesity, links obesity with tumour growth (Honma *et al.*, 2002; McMurphy *et al.*, 2014). The levels of this hormone are raised significantly in obese subjects, which suggests that obesity may associate with leptin resistance (Van Kruijsdijk *et al.*, 2009). Several publications reported the important role of leptin in the development of a large variety of malignancies by increasing the expression of anti-apoptotic proteins, inflammatory markers and angiogenic factors (Choi *et al.*, 2005; Sharma *et al.*, 2006). These processes enhance cancer cell survival, proliferation and migration (Olefsky and Glass, 2010; Booth *et al.*, 2015). However, a direct cause for leptin in accelerating tumour growth is uncertain (Mendonsa *et al.*, 2015). Leptin mediates its effect by binding to multiple splicing isoforms of the leptin receptors (ObRs), and these receptors are ubiquitously express in all peripheral tissues (Glasow *et al.*, 2001). They function through the activation of Janus kinases (JAK) and STATs, especially the JAK2-STAT3 signal transduction pathway. In addition, PI3K and mitogen-activated protein kinase (MAPK)/ signalling pathways also support neoplastic growth properties of leptin (Ray and Cleary, 2017). The role of leptin and its receptors in growth and proliferation has been extensively explored in different cancers such as breast, prostate, colon cancer (Malvi *et al.*, 2018). Additionally, the fact that leptin production is markedly elevated in obesity, generates much interest around the role of this adipokine in the pathogenesis of obesity (Duong *et al.*, 2017). Leptin plays an important role in modulating hepatic lipogenesis by inhibiting the expression of basic enzymes in the fatty acid synthesis pathway, and induces fatty acid oxidation in muscle through activation of a critical energy sensor 5’ AMP-activated protein kinase (Cao, 2014). Studies *in vitro* have shown that leptin has a mitogenic and anti-apoptotic effects

on cancer cells. However, these actions may depend on the specific types of cancer cells. Importantly, supressing MAPK signalling pathways inhibits these actions, indicating that this pathway underlies the growth-promoting effect of leptin (Van Kruijsdijk *et al.*, 2009). Animal models of obesity show that HFD fed mice has significant up-regulation of leptin concentrations and enhanced melanoma tumour growth and progression (Malvi *et al.*, 2015). Furthermore, investigators have shown a significantly higher concentration of leptin in melanoma patients with positive sentinel lymph node involvement (Oba *et al.*, 2016).

1.6.5 Monocyte chemo-attractant protein 1 (MCP-1)

Monocyte chemoattractant protein 1 (MCP-1) (also referred as chemokine C-C motif ligand 2, CCL2), a well-known chemotactic chemokine which is not only express by immune cells, but also expresses by many types of malignant and stromal cells (Li *et al.*, 2013). Adipocytes are an important player in the tumour microenvironment and are considered as a main producer of MCP-1 which causes adipose tissue inflammation (Correa *et al.*, 2017). Consistent with this notion, mice fed a HFD had expansion of adipose tissue and elevated plasma MCP-1 after 4-weeks feeding (Chen *et al.*, 2005). Increased CCL2 secretion from adipocytes may be associated with the early phase of tumorigenesis promoting localised inflammation through adipose tissue macrophages (Arendt *et al*. 2013). Several published studies indicate the main role of CCL2 and its receptor CCR2 in tumour growth and progression is by sustaining cancer cell proliferation, stimulating cancer cell migration and invasion, recruitment of TAMs and MDSCs, and inducing deleterious inflammation and angiogenesis (Youngs *et al.*, 1997; Conti and Rollins, 2004; Izhak *et al.*, 2012).Given these influential roles in tumour growth, the CCL2 –CCR2 signalling pathway appears as a promising target for the development of novel anticancer therapeutics (de Lima-Salgado *et al.*, 2011). In melanoma, overexpression of CCL2 enhances vascular permeability and angiogenesis, and increases recruitment of mononuclear cells and M2 polarized macrophages (Jung *et al.*, 2015). Furthermore, conditioned medium from cancer associated adipocytes showed an increase in CCL2 and IL-6 production than that from the normal breast adipocytes (Fujisaki *et al.*, 2015).

1.7 A role for Fatty acid and its receptor (GPR120) in cancer pathology

Enlarged adipose tissues during HFD -induced obesity lead to excessive realise of fatty acids (FAs) and pro-inflammatory cytokines (Boden, 2008). FAs basically is a lipid molecule consisting of hydrocarbon chains terminating with carboxylic acid groups and they have an even number of carbon atoms (Currie *et al.,* 2013). FAs can be either saturated or unsaturated, and they have an essential role not only in membrane synthesis and energy metabolism, but also in several pathogenic condition associated with fatty acids metabolism (Ying and Zhu, 2016).

Fatty acids mediate their biological effects through binding to Fatty Acid receptor 4 (FFAR4) also known as GPR120, this receptor belongs to the G-protein-coupled receptors family (GPCRs) which comprise an important family of cell-surface receptors (Kleemann *et al.,* 2018). GPR120 plays a crucial role in the regulation of various physiological processes such as insulin sensitivity, macrophage inactivation, adipocyte differentiation, glucose homeostasis and glucagon-like peptide-1 production (Wang *et al.,* 2019). GPR120 is widely expressed by different tissues and cells such as adipose tissue, macrophages, intestinal and pancreatic tissue (Anbazhagan *et al.*, 2016). However, expression was significantly higher in adipose tissue and macrophages of obese compared with lean subjects (Wu *et al.,* 2013). These distributions suggest that GPR120 has multiple roles in the homeostatic regulation of metabolism and inflammation (Ichimura *et al.*, 2014). Increasing evidence shows that GPR120 has an important role in tumour formation, migration, and metastasis (Houthuijzen, 2016). In contrast to its contributions to metabolic process, GPR120 seems to have opposing functions in cancer pathology. Activation of GPR120 signalling by fatty acids enhances the survival of murine intestinal tumour cells under serum free conditioned medium (Katsuma *et al.*, 2005).

Published data also showed that GPR120 enhances cell migration of colorectal carcinoma cells *in vitro* and in xenograph mouse models (Wu *et al.,* 2013). It has been suggested that the action of GPR120 on cell migration are mediated by matrix metalloproteinase 2 (MMP-2), as cell lacking GPR120 displayed low levels of MMP-2 (Fukushima *et al.,* 2016). In addition to its effect on cell migration and metastasis, other tumourgenic functions have been attributed to GPR120 signalling pathways (Zhang and

Qiu, 2019). GPR120 signalling pathway in colorectal cancer (CRC) induced angiogenesis of human umbilical vein endothelial cells due to the production of VEGF, IL-8, and cyclooxygenase-2 derived PGE2 production by CRC (Wu *et al.*, 2013). Additionally, *in vivo* studies showed that activation of GPR120 expressed by splenic Macrophages could protect tumour cells from chemotherapy induced DNA damage (Houthuijzen, 2016).

1.8 The role of a high fat diet on tumour growth

In general, cells use fatty acids either from diet or endogenous sources. According to previous studies, consuming a diet rich in fat could be a main factor in tumour initiation and progression (Long *et al.*, 2018). Murine experiments showed that HFD feeding induced poorly differentiated carcinomas in transgenic mouse prostate cancer model and up-regulated growth factors responsible for cell proliferation and angiogenesis like vascular endothelial growth factor (VEGF), which facilitates tumour growth and reduce survival rate of these mice (Cho *et al.*, 2015). Published data found that excess fat intake induced an increase in M2 MΦs recruitment into the tumour microenvironment and reduced cancer cell apoptosis which enhance cell proliferation, angiogenesis and thereby accelerate tumour growth in a breast cancer mouse model (Zhu *et al.*, 2016). Additionally, chronic consumption of dietary rich fat showed an increase in the expression of macrophages markers like toll-like receptor (TLR-4) and complement receptor 4 (CR4) as well as up-regulation of pro-inflammatory adipokines which provide evidence linking HFD feeding and mouse colon cancer (Day *et al.*, 2013). Tessitore *et al.* (2017) demonstrated that long term feeding of a HFD elevated IL-6, TNF-α and IL-1β and enhanced liver tumour growth in obese mice. Moreover, subcutaneous injection of the murine melanoma cancer cell (B16-F10) induced larger tumours in mice fed on a HFD compared to those fed on normal diet (Jung *et al.*, 2015; Malvi *et al.*, 2015; Pandey *et al.*, 2012). In mouse models of HFD-induced obesity, a HFD induced melanoma cell proliferation, and anti- apoptosis (Jung *et al*. 2015), and up-regulated caveolin1 and fatty acid synthase (FASN) (Pandey *et al.*, 2012), resulting in larger tumours. Moreover, intravenous injection of B16-F10 in obese mice induces lymph node, lung and bone metastases of melanoma tumours (Mori *et al.*, 2006). Interestingly, caloric restriction has been shown to reverse melanoma tumour aggressiveness in mice (Malvi *et al.*, 2015).

1.9 Cellular fatty acids metabolism in cancer

Cancer is fundamentally a disorder of cell growth and proliferation, which requires cellular building blocks, such as nucleic acids, proteins, and lipids. Cancer cells often have perturbed metabolism that allows them to accumulate metabolic intermediates as sources of these building blocks (Currie *et al.*, 2013). Alteration in lipid metabolism has been increasingly recognised as a hallmark of aggressive cancers (Zhang and Du, 2012). Although cancers are greatly diverse in their types and aetiology, cancer cells frequently share the same attributes of metabolic abnormalities (Beloribi-Djefaflia *et al.*, 2016). Malignant cells reprogramed their metabolic activities to support their abnormal demands for proliferation and survival (Cairns *et al.*, 2011). A large body of evidence supports the notion that oncogenic signalling pathways regulate lipid metabolism in various cancer types and importantly are involved in initiation and progression of these tumours (Liu, 2006). Lipids are a group of water-insoluble molecules which are divided into two main groups: including (phospholipids, glycolipids, sphingolipids, and sterols), and fat such as triglyceride (TG). Sterols also include cholesterol, sex hormones and VitD3. These lipids are digested and absorbed in the intestine and then are metabolised in the liver and stored in adipocytes, used by other tissues when required (Long *et al.*, 2018).They have an influential cellular role in metabolism as energy sources. Furthermore, cells use lipids from the bloodstream either in the form of free fatty acids or as low-density lipoprotein. These lipids can be acquired from dietary lipids, and carbohydrate-derived fatty acids which are synthesized in the liver or in adipocytes. FAs are the main building blocks in triacylglycerides synthesis and the main source for energy storage (Santos and Schulze, 2012). FAs metabolism is a key process that influences various cellular pathways and properties including cell signalling, energy processing and membrane fluidity (Liu, 2006).

FAs are also required for the production of phosphoglycerides, which, together with cholesterol, can be used for cell membranes synthesis. However, deregulated fatty acids metabolism favouring excess lipid biosynthesis and deposition eventually predisposes the body to metabolic disorders and carcinogenesis (Tang *et al.*, 2018). Malignant cells exhibit alterations in their lipid metabolism. For example, abnormalities in lipid metabolism can significantly affect the amount of structural lipids that are used in membrane formation and the synthesis and degradation of lipids involved in energy homeostasis and the availability of lipids with signalling functions (Santos and Schulze,

2012). In addition to diet, neoplastic cells are endogenously and actively use *de novo* synthesised fatty acids, whereas most normal cells preferentially use exogenous fatty acids to meet their need. However, tumour cells are also able to use exogenous cholesterol, by Low-density lipoprotein (LDL) (Beloribi-Djefaflia *et al.*, 2016).

Moreover, various types of cancer showed up-regulation in the activity of many enzymes involved in fatty acid synthesis such as ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). Therefore, the rate of lipogenesis may be increased in transformed active cells (Tennant *et al.*, 2010). However, the cellular mechanism by which FASN is up-regulated in cancer cells is not fully understood (Pandey *et al*., 2012). The committed step in *de novo* fatty-acid biosynthesis requires the conversion of acetyl-coA to malonyl-CoA by acetyl-coA carboxylase (ACC). Malonyl CoA and acetyl-coA are further processed by the multifunctional enzyme fatty-acid synthase (FASN) to the saturated fatty acid palmitic acid which is then converted into stearic acid and then into Triglyceride (TG), an important lipid molecule that plays a major role in metabolism process, thermo-modulation, and supporting absorption of fat-soluble vitamins. These processes occur mainly in the liver and adipose tissues which provides the cells with energy to support rapid cell proliferation (Mounier *et al.*, 2014). In addition, fatty acids can also be mobilised by a series of mitochondrial β-oxidation processes leading to a breakdown of fatty acid into acetyl-CoA which then enters the tricarboxylic acid cycle to aid ATP generation (Tang *et al.*, 2018). Noticeably, fatty acid activation by acyl-CoA synthetase (ACS) is the common initial step in both *de novo* lipogenesis and fatty acid oxidation (**Figure 1-6**).

It is important to mention that reactivation of *de novo* lipid synthesis or the development of a "lipogenic" phenotype by tumour cells has been demonstrated in several studies (Peck and Schulze, 2016; Lisec *et al.*, 2019). Some types of cancer including breast and prostate showed an increase in FASN expression, which suggests that FASN may play a role in cancer pathogenesis (Kwan *et al.*, 2014). Interestingly, Obesity induced metabolic alterations by increasing the expression of FASN in a melanoma mouse model, which indicates that FASN may act as tumour promoter (Pandey *et al.*, 2012). Furthermore, inhibition in fatty acid synthase activity (Seguin *et al.*, 2012), and deactivating lipolytic enzyme monoacyl-glycerol lipase (Nomura *et al.*, 2010), reduces endogenous palmitic acid/fatty acid levels and attenuates melanoma tumour growth. Furthermore, *in vitro* and *in vivo* experiments showed that ATP-citrate lyase was

required for tumour cell growth (Bauer *et al.*, 2005; Hatzivassiliou *et al.*, 2005), and chemical inhibition of Acetyl-CoA carboxylase (ACC) causes growth arrest and apoptosis in prostate cancer cells, suggesting that FAs have a significant contribution to both tumorigenesis and metastasis, and may be a good target for cancer therapy (Hu *et al.*, 2013).

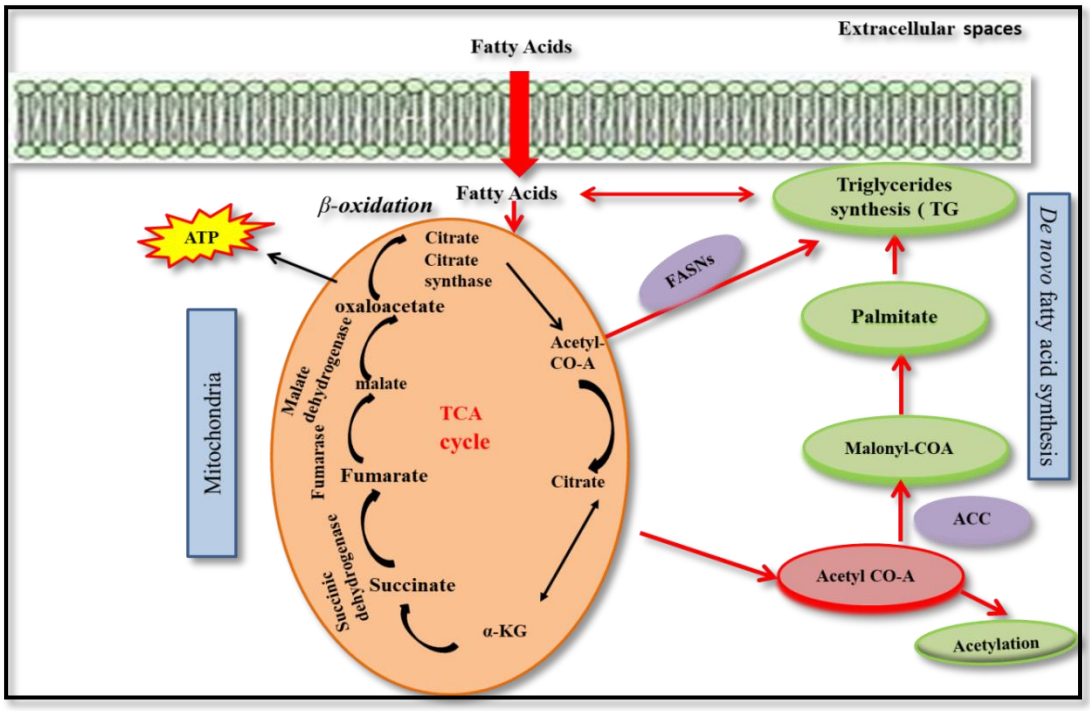


Figure 1-6: Cellular fatty acid metabolism in cancer cells.
FAs generated either from *de novo* process by carboxylated Acetyl-CoA (ACC) to malonyl-CoA which is then converted by FA synthase (FASN) to generate palmitate before being converted to triglyceride (TG) or by mitochondrial β-oxidation of acetyl-CoA (ACC) by FASN in Tricarboxylic acid cycle (TAC).

1.10 Obesity-derived systemic complications

Adipose tissue is a matrix containing a vast variety of cells including mostly adipocytes, pre-adipocytes, endothelial cells, blood vessels and immune cells (Jung and Choi, 2014). The capacity of adipose tissue to store TG as lipid droplets leads to an increase in the number of adipocytes (hyperplasia), or an enlargement in the size of adipocytes (hypertrophy). Hypertrophic expansion of adipose tissue is the main feature of obesity related metabolic stress and is the major cause in adipose tissue dysfunction (Spalding *et al.*, 2008). Metabolism of storage TG through lipolysis leads to release of free fatty acids into the circulation and transport to other tissues such as the liver, heart, and

skeletal muscle to be used as an energy source. However, these tissues are less able to store lipid than adipose tissue which induces accumulation of toxic fatty acid metabolism and leads to inflammation and impairs insulin sensitivity (Ramos-Nino, 2013). Furthermore, in an obese setting, adipocytes showed activation of some transcription factors such as *NF-κB*, signal transducer and activator of transcription 3 (STAT-3) and hypoxia-inducible factor-1 alpha (HIF-1α) (He *et al.*, 2011; Jiang *et al.*, 2013). These TFs enhance inflammatory gene targets, such as prostaglandins, cyclooxygenase-2 (COX-2), cytokines (tumour necrosis factor- alpha (TNF-α), interleukin (IL-1, IL-6), chemokines (CXC-chemokine ligands (CXCLs) and chemokine receptors, which leads to a chronic "low-grade" inflammatory response (Greenberg and Obin, 2006; Kabir *et al.*, 2014). The inflammatory microenvironment in adipose tissue could predispose obese individuals to develop metabolic syndromes, including insulin resistance, type 2 diabetes and obesity-related cardiovascular disease (Lumeng and Saltiel, 2011; Jung and Choi, 2014; Gregor and Hotamisligil, 2011). A chronic inflammatory state is regarded as a critical mediator for both obesity and cancer (Van Kruijsdijk *et al.*, 2009). Additionally,adipose tissues provide systemic endocrine factors, such as adipokines and pro-antigenic factors,which favour both melanoma initiation and progression (Nieman *et al.*, 2011; Rivera-Gonzalez *et al.*, 2014).

1.11 Obesity and cancer related inflammation

Different mechanisms have been proposed to explain how obesity drives cancer growth and progression. Chronic inflammation, a phenotype associated with obesity, is considered as a major factor contributing to cancer growth and development (Kolb *et al.*, 2016). Obesity-related inflammation is associated with increases in cancer prevalence and mortality, given inflammation sets the stage for malignancies (Wolin *et al.*, 2010). Inflammation is a chief player in cancer pathogenesis, as multiple immune cells, pro-inflammatory mediators and growth factors are present within the tumour microenvironment (TME). Their interaction facilitates cell proliferation, promotes angiogenesis, and enhances cancer cell migration and metastasis (Hanahan and Coussens, 2012). During inflammation many promoters, whether directly or indirectly, promote cell proliferation, recruit inflammatory cells, increase production of reactive oxygen species (ROS) and thereby inducing oxidative DNA damage, and inhibit DNA repair. Subversion of cell death and/or repair programmes occurs in chronically

inflamed tissues, leading to replication of DNA and cell proliferation in cells that have lost normal growth control (Reuter *et al.*, 2010). Notably, not all inflammatory responses are responsible and induce cancer development. For example, accumulation of $CD8^+$ T cells and natural killer (NK) during acute inflammation triggers anti- tumour responses (Kim and Karin, 2011). However, these cells are less abundant at sites of chronic low-grade inflammation (Deng *et al.*, 2016). Several mechanisms have been suggested to explain the association between cancer and obesity. These include expansion of adipose tissue during obesity which leads to induction and release of fatty acids which act as a danger associated molecular pattern (DAMP) that activates toll-like receptor (TLR-4) expressed by macrophages.This leads to up-regulation of nuclear factor kappa B- (NF -κB) dependent expression of pro-inflammatory genes such as IL-6, TNF-α and cyclooxygenase-2 (COX-2) and increases the inflammatory biomarker C-reactive protein (CRP) which leads to a low-grade inflammation (Iyengar *et al.*, 2016). Furthermore, obesity alters adipose tissue (a powerful tumour neighbourhood) by increase production of adipokines and chemokines that affecting multiple systemic processes including the TME. In particular, the secretion of plasminogen activator inhibitor-1 (PAI-1), IL-6, CCL2, Adiponectin, leptin, osteopontin, TNFα and Resistin significantly impact several aspects of tumour initiation and progression. Additionally, cancer-associated adipocytes (CAAs) present within the TME fuel tumours and promote their proliferation. Furthermore, adipose tissue enhances tumour angiogenesis by providing adipose-derived stem cells (ASCs) which can become cancer-associated fibroblasts. Finally, obesity-associated metabolic syndrome induces systemic insulin resistance, which promotes up-regulation of circulating insulin and insulin-like growth factors impacting tumour growth (Deng *et al.*, 2016).

1.12 The role of Toll like receptor-4 (TLR-4) in cancer

Toll-like Receptor-4 (TLR-4) is type I transmembrane protein and a member of the Toll-like Receptor family (Ran *et al.*, 2019). This receptor belongs to the pattern recognition receptor (PRR) family and it is expressed on the cell surface on both hematopoietic and non- hematopoietic cells, including endothelial cells, cardiac myocytes and cells of the central nervous system (CNS) (Molteni *et al*., 2016). The activation of this receptor leads to an intra-cellular signalling pathway NF-κB activation and thereby production of pro-inflammatory cytokines which is responsible for activating the innate immune system (Rogero and Calder, 2018). TLR-4 can bind pathogen-associated molecular patterns (PAMPs), such as (LPS) and endogenous damage-associated molecular patterns (DAMPs) which are released during infectious and non-infectious inflammatory conditions (Awasthi, 2014). Chronic infections and inflammatory conditions are known to promote carcinogenesis, evidence from published reports revealed that increased expression and activity of TLR-4 in chronic infectious and inflammatory conditions is associated with cancer progression (Oblak and Jerala, 2011). Inflammation has an undisputed role in cancer development and progression, TLR-4 is expressed not only on immune cells, but also on tumour cells such as human lung cancer cell lines A549 and H460 (Zhan *et al.*, 2014), human cervical squamous epithelial cells (Yu *et al.*, 2010), and colon cancer cells (Wang *et al.*, 2010). Previous studies have revealed that adipose tissue expansion during obesity dysregulated the production of pro-inflammatory or inflammatory cytokines, obesity could be considered as a chronic inflammatory disease triggered by fatty acids release which is act as DAMP molecules (Jounai *et al.*, 2012). Free fatty acids can serve as ligands for the toll-like receptor-4 (TLR-4) and induce the secretion of cytokines by macrophages, thereby modulating inflammation of adipose tissue (Shi *et al.*, 2006).

1.13 The role of Vitamin D3 in obesity

Vitamin D3 is a fat-soluble steroid hormone, obtainable mainly from skin after exposure to ultraviolet B sunlight (UVB, 290–320 nm), which represents nearly 80% of all systemic vitamin D3. Alternatively, it can be obtained through diet from animals, (cholecalciferol (D3), or plants (ergocalciferol D2) sources as well as from dietary supplementation (Cipriani *et al.*, 2014). VitD3 synthesised by conversation of epidermal 7-dehydrocholesterol (cholecalciferol, pro-vitamin D3) in to previtamin-D3 (pre-D3) via ultraviolet radiation (UVR) in photochemical process. According to the classical pathway, VitD is hydroxylated in the liver by vitamin D 25-hydroxylase (CYP2R1 and CYP27A1) into 25-hydroxy-vitamin D (25(OH) D) (calcidiol), which is the major circulating form of vitamin D in serum. This is then converted in the kidney by 25(OH)D 1α-hydroxylase (CYP27B1) into a biologically active metabolite form (calcitriol) 1,25-dihydroxyvitamin D1,25(OH)2D (**Figure 1-7)** (Cipriani *et al.*, 2014). Notably, the concentration of formed VitD3 depends on several influential factors such as the intensity and duration of UVB exposure, skin pigmentation levels, age and obesity (Bikle, 2014). According to UK Medicines Information (UKMI), people are diagnosed with VitD3 deficiency when the concentration is below 30 nmol/L (<12 ng/mL), while individuals with levels ranging from 30–50 nmol/L (12–20 ng/mL) are potentially at risk. Vitamin D3 levels ≥50 nmol/L (≥20 ng/mL) is considered optimal in adults. However, levels above >125 nmol/L (>50 ng/mL) may associate with harmful effects (UKMI, 2017). VitD3 exerts its action through binding to the vitamin D receptor (VDR), an endocrine member of the steroid hormone nuclear receptor family (Jeon and Shin, 2018). In addition to the classical action of VitD3 in regulating calcium absorption, Vitamin D3 also plays a crucial role in the regulation of several biological processes such as glucose homeostasis, insulin secretion and inflammation associated with obesity (Zakharova *et al.*, 2019).

Several studies reported that serum concentrations of VitD3 show an inverse correlation with fat mass and BMI (Vimaleswaran *et al.*, 2013; Lenders *et al.*, 2009). In obesity, VitD3 induces an increase in the expression of insulin receptors in peripheral cells, and neutralises the systemic immune response by modulating the production and action of pro-inflammatory cytokines which affect insulin sensitivity, and systemic inflammation (El-Hajj Fuleihan, 2012). VitD3 modulates the expression of genes responsible for the production of leptin and Adiponectin, important players in metabolic processes

(Mantzoros *et al.*, 2011). Earlier, it was shown that dietary administration of VitD3 led to reduce diabetes and increased $FoxP3^+$ T cells in non-obese diabetic mice (Driver *et al.*, 2008). Additionally, the excessive accumulation of fat induces deactivation of alpha-hydroxylase, a specific enzyme responsible for biotransformation of calciferol in kidney which leads to the accumulation of inactive forms and reduces vitamin D3 bioavailability (Zakharova *et al.*, 2019). During obesity, a low-grade inflammatory state, vitamin D3 mediated anti-inflammatory action occurs by inhibiting the secretion of pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and IL-12 (Christakos *et al.*, 2015). VitD3 modulates visceral adipose tissue inflammation, whilst not reducing subcutaneous fat tissue inflammation (Abbas, 2017). Epidemiological studies revealed decreases in TNF-alpha levels and a reduction in adipose tissue inflammation in overweight and obese patients with normal VitD3 concentrations (Olszanecka-Glinianowicz *et al.*, 2011). Vitamin D3 counteracts systemic inflammation by suppression of mitogen activated protein kinase signalling pathways and NF-*κB* activity (Karkeni *et al.*, 2018), down-regulation of toll-like receptor (TLRs) expression, a block of dendritic cell differentiation and inhibition of lymphocyte proliferation (Calton *et al.*, 2015).

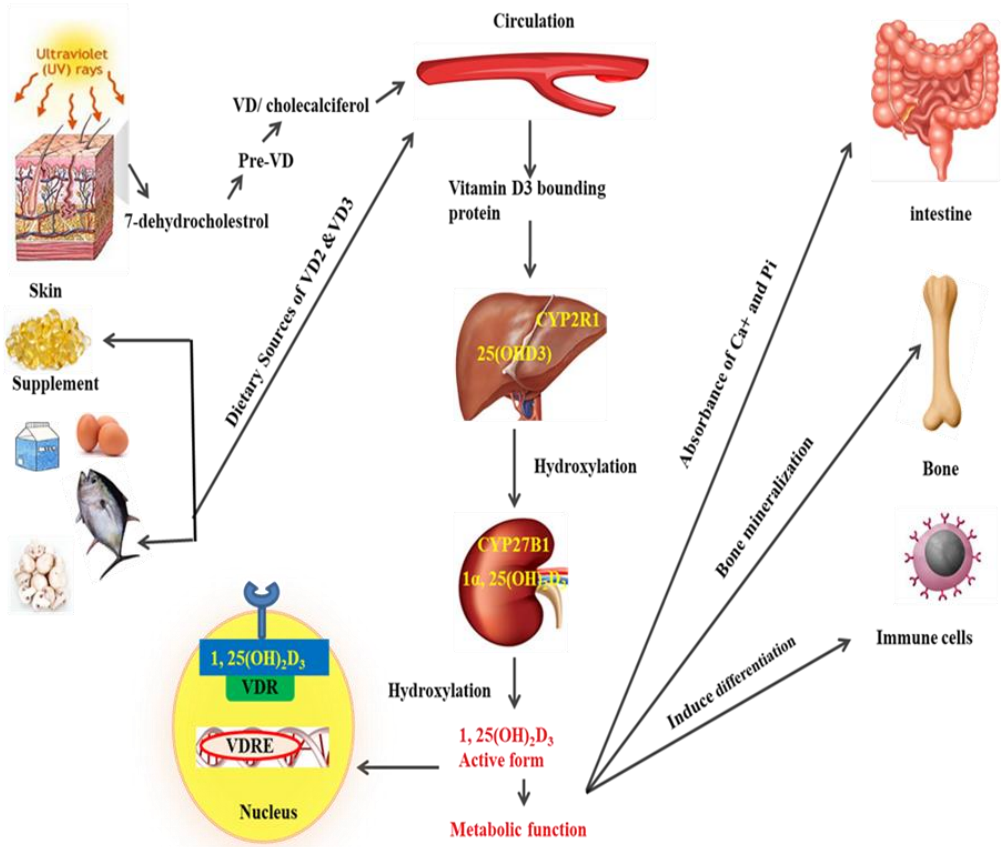


Figure 1-7: Basic metabolism and synthesis of vitamin D3.

The stored 7-dehydrocholesterol is converted to pre-Vitamin D3 or pre -calciferol after exposing the skin to certain wavelengths of ultraviolet B (UVB) rays from the sun. Cholecalciferol is formed from vitamin D3 which is formed after isomerization of pre-vitamin D3. Vitamin D2 or D3 from the diet and supplements will be combined to form chylomicron vitamin D which is then bound to binding protein (DBP) in liver. Then the Vitamin D3 in the liver is metabolised by 25-hydroxylase (CYP2R1), a so-called calcidiol. In the kidney a second hydroxylation event occurs because of 1α-hydroxylase (CYP27B1) activity that forms 1, 25-(OH) 2 D3 (calcitriol) which is the active form of Vitamin D3. Following activation, the metabolite, 1, 25-(OH) 2 D3 (calcitriol) acts as a ligand for the vitamin D receptor (VDR) resulting in altered genes expression.

1.14 Dysregulating of vitamin D3 metabolism in cancer

Vitamin D3, which is traditionally known as an essential nutrient, also has a classic role in bone remodelling by regulating calcium and phosphate metabolism. However, accumulating evidence showed that many types of tumour dysregulate the function and metabolism of VitD3, conferring resistance to the antitumorigenic action of VitD3 which leads to cancer development and progression (Jeon and Shin, 2018). It has been classically considered that calcitriol (an active form of VitD3), producing enzyme CYP27B1 is expressed in the kidney, and that vitamin D3 receptor and CYP24A1 enzyme, which mediate calcitriol function and degradation, are expressed only in certain tissues such as intestine, bone, and kidney. However, these proteins are widely expressed in other tissues (Bikle, 2010), suggesting that VitD3 could be locally regulated and most tissues could be targets for VitD3. Importantly, this local regulation of VitD3 is dysregulated in various cancer cells, which contributes to the resistance to anti- cancer action of VitD3 (Bikle, 2016). The vitamin D3 receptor (VDR) is highly expressed in most cells; however, this expression is significantly down regulated during tumour development and progression in different cancer types. Importantly, up-regulation of VDR expression was inversely correlated with tumour aggressiveness and cancer death in prostate cancer and a better prognosis and survival rate in lung cancer (Hendrickson *et al.*, 2011; Srinivasan *et al.*, 2011). Thus, VitD3 may play a beneficial role in cancer prevention. However, cancer cells manipulate their ability to expresses VitD3 receptors and thereby escape from tumour-suppressive actions of VitD3 (Jeon and Shin, 2018).Similarly, the expression of CYP27B1 (a metabolic enzyme responsible for conversion of calcidiol into calcitriol) is negatively associated with the progression of several tumours such as skin, colon and lung (Brozyna *et al.*, 2011; Hsu *et al.*, 2011). Interestingly, pro-inflammatory cytokines such as IL-6 and TNF-α and activation of Toll-like receptors (TLRs) decrease CYP27B1 levels in colon cancer cells, suggesting that inflammation within tumour microenvironments could be a contributing factor that down-regulate CYP27B1 expression during tumour growth. However, the mechanism responsibly for this reduction remains unknown (Hummel *et al.*, 2014) (**Figure 1-8**). Furthermore, cancer cells up-regulate the expression of CYP24A1 (a metabolic enzyme responsible for degradation of calcidiol and calcitriol) to reduce local concentrations of calcitriol, suggesting that malignant cells escape from the anti-cancer effects of calcitriol by up-regulation of CYP24A1 which leads to a decrease in calcitriol

expression (Albertson *et al.*, 2000) (**Figure 1-8**). The overexpression of CYP24A1 has been reported in many tumours such as ovarian, cervical, basal cell carcinoma and lung, and it is associated with poor prognosis and resistance to vitamin D-based therapy (Lopes *et al.*, 2010; Matusiak and Benya, 2007). Supporting the oncogenic effect of CYP24A1, the suppression CYP24A1 inhibited tumour growth and boosts the anti-tumorigenic action of calcitriol in breast and lung tumours, suggesting that CYP24A1 could be a promising target for anti-cancer therapy (Chen *et al.*, 2011). Epidemiological, clinical and *in vitro* studies reported that therapeutic interventions targeting dysregulated vitamin D3 metabolism or activity could be used as a promising approach in cancer prevention (Jeon and Shin, 2018). Published studies revealed that VitD3 may play an important role in prevention and treatment of different extra-skeletal diseases such as cancer (Ebeling *et al.*, 2018). Diverse mechanisms have been proposed to explain the beneficial action of VitD3 in cancer prevention. Published data suggested that VitD3 may play an influential role throughout tumourgenesis, from initiation to metastasis as well as cell interactions within the tumour microenvironment (TEM). These mechanisms include regulation of cellular interactions pathways, anti-inflammatory, and modulate cellular behaviours such proliferation, differentiation and apoptosis (Giammanco *et al.*, 2015).

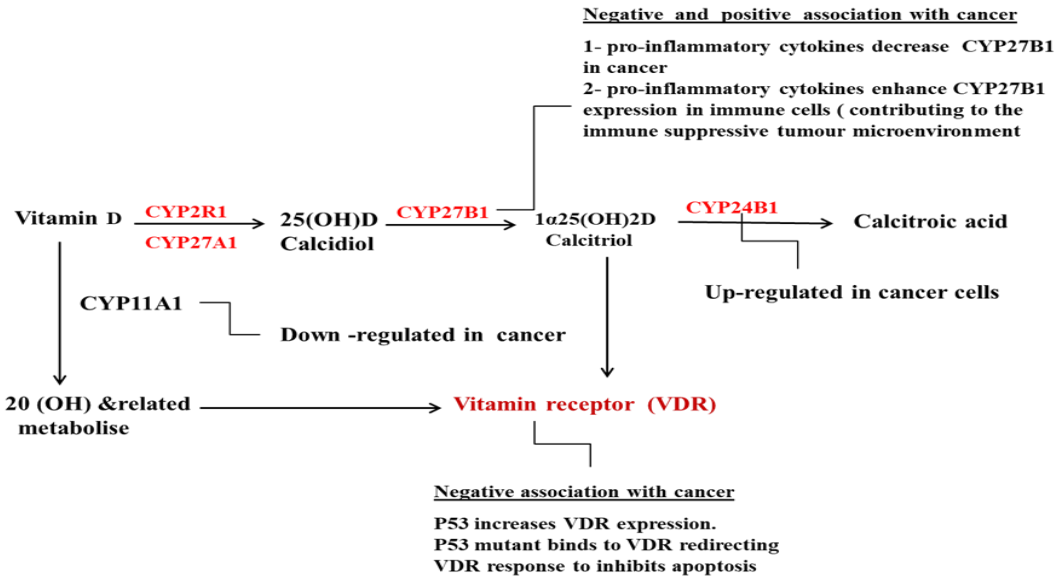


Figure 1-8: Dysregulation of Vitamin D3 metabolism in cancer.
Cancer cells down–regulate the expression of VDR, up-regulate (CYP24A1) enzyme mediate degradation of calcidiol and calcitriol and down-regulate CYP27B1enzyme associated with conversation of calcidiol into calcitriol (active form of VitD3).

1.15 Amis

The overall aim of the present study was to investigate the effect of obesity related inflammation on melanoma tumour growth and the tumour immune microenvironment in a hyperlipidaemic mouse model. This study also tested the putative anti-inflammatory action of a Vitamin D3 supplemented diet in obesity induced tumour development.

Finally, the study also aimed to determine the frequency of immunosuppressive tumour derived lymphoid (T-cells) in *LDLR*$^{-/-}$ tumour bearing mice spleen fed different diets.

1.16 Hypotheses

1- High Fat Diet (HFD) induced obesity influences tumour growth and modulates the tumour microenvironment (TME) in a low-density lipoprotein receptor deficient (*LDLR*$^{-/-}$) mouse model (prone to developing metabolic syndrome).

2- Obesity induced adipose tissue dysfunction alters cytokine and chemokine profile.

3- The anti-inflammatory action of Vitamin D3 impacts melanoma tumour growth and the TME in obese *LDLR*$^{-/-}$ mouse models.

1.17 Objectives

Before establishing animal studies, a series of *in vitro* experiments was done to investigate the effect of fatty acid (either purified or present in the serum of HFD fed animals) and Vitamin D3 (either supplemented or exogenously added to HFD serum) on phenotypic and tumorigenic actives of two important cell types in the tumour microenvironment (TME), murine macrophages (modelled using the J774 cell line) and mouse skin cancer (using the orthotopic B16-F10 melanoma cell line) according to the following:

1- Investigate the effect of different concentrations of purified fatty acids, or serum from HFD or Control Diet (CD) fed mice on B16-F10 cells. This includes assays of viability (MTS and crystal violet assays), migration (wound healing assays), lipid inclusion (Oil red O staining), changes in chemokine and cytokine secretion (proteome profile array and ELISA) as well as expression of specific receptors such as Leptin (ObRs) and fatty acid receptors (GPR120) (Real-time PCR and Western blotting).

2- Identify the action of fatty acids at different concentrations and cell conditioned media from B16-F10 tumour cells on murine macrophages (J774). Phenotypic changes were measured using qPCR for the expression of Arginase-1 and iNOS as markers for M1/M2 polarisation.
3- Investigate proliferation, migration and secretion of cytokines and chemokines (TNFα and CCL2) in J774 macrophages treated with different concentrations of fatty acids using crystal violet assay, wound healing and ELISA, respectively.
4- Investigate the effect of HFD + VitD3 at different concentrations (1 and 10μM) and a HFD supplemented with VitD3 on B16-F10 mitochondrial cell activity, lipid inclusion ability and migration using MTS, Oil red O staining and wound healing, respectively.

To establish a model of obesity *in vivo*, *LDLR*$^{-/-}$ mice will be given formulated diets containing high fat and sugar (HFD) for different durations (two and ten weeks) before subcutaneous injection of B16-F10 (murine melanoma cell line) into syngeneic recipients to initiate the tumour model. Furthermore, these mice will also be fed on a HFD supplemented with VitD3 to identify its anti- inflammatory action in obesity related inflammation in *LDLR*$^{-/-}$ tumour bearing mice. Therefore, the specific objectives of *in vivo* study were:

1- To confirm by measurement of body weight that dietary rich fat induces an increase in body weight in *LDLR*$^{-/-}$ mice.
2- Phenotypic characterisation of tumours implanted in obese *LDLR*$^{-/-}$ mice fed on a HFD, CD and a HFD containing VitD3 (Macroscopic examination and measurement of tumour weight and size).
3- Flow cytometry was used to quantify and identify splenocytic T-reg populations using specific markers ($CD4^{+}CD25^{+}FOXP3^{+}$).
4- Measurement of adipocytokine and chemokines secretion (Leptin, IL-6, IL-10, TNF-α, CCl2) in mice sera and investigate the expression of Leptin receptor (ObRs) and GPR120 in tumour tissues using ELISA assays and qPCR, respectively.
5- Determine (using the XL proteome profiler array) the ability of diet–induced obesity to alter chemokine profiles in *LDLR*$^{-/-}$ tumour bearing mouse sera

Chapter 2

Materials and Methods

Materials and Methods

2.1 Animals

The experiments were carried out using specific pathogen-free genetically modified mice. They were bred in the Preclinical Research Facility (PRF), at the University of Leicester and used with authority from the Home Office under the supervisor's project license P43308E3B, with approval of the institutional animal welfare, and ethical review board. The supervisor who held a personal license and was certified by the named training officer to be competent to perform the subcutaneous injections.

Low Density Lipoprotein Receptor deficient mice (*LDLR*$^{-/-}$) on C57BL/6 background (Ldlr^{tm1Her}/J; Jackson Laboratories) were used in this study as a model of familial hypercholesterolemia and initially were part of a study conducted during a British Heart Foundation-funded grant period at University of Leicester and University of Sheffield. They were maintained since the arising publication (Steiner *et al.*, 2014), and were available as part of this work. All mice were housed in a controlled environment with a 12 hours light/dark cycle at 22 ^{0}C.

2.2 Diets

Different types of diet were used in this study according to their purpose. Mice were maintained on a 5LF2 diet (**Table 2-1**), and randomly assigned to one of two diets groups as follows: [high fat (58R3)] (**Table 2-1**), [High fat supplemented with Vitamin D3 (58R3+VitD3)*] and [low fat and low estrogen diet (58R1)] was used as control diet (CD) (**Table 2-1**). *LDLR*$^{-/-}$ mice were given these diets for different period 2- and up to 10- weeks prior injection with murine melanoma cell line (B16-F10). The change in body weight was monitored and measured weekly.

Table 2-1: Composition of diets used in this study

Nutritional profile	Normal Chow Diet (Erodent diet %14 (5LF2)	Low Fat Diet (58R1)	High Fat Diet (58R3)
% protein	14.3%	14.8	20.2
% Fat	5.8 %	4.8	35.8
% carbohydrate	65	73.9	35.0
Soybean meal (Phytoestrogen source)	Up to 20%	Not included	Not included

*** HFD (58R3) differing in the content of admixed Vitamin D3 (1 IU/g *vs* 10 IU/g).**

2.3 Cell lines

2.3.1 Murine Melanoma cell line (B16-F10)

The cell line was used in this study is B16, a murine melanoma cell line obtained from C57BL/6 mice (kindly provided by Professor Steven Todryk, University of Northumbria). The B16-F10 cell line was derived from the parental B16- F1 line which was chosen according to their ability to form lung colonies *in vivo* after intravenous injection and subsequently established *in vitro* after 10 cycles (B16-F10) of lung colony formation (Danciu *et al.*, 2013), B16 forms a palpable tumour in 5 to 10 days when injected subcutaneously. These cells have traditionally been described as low-immunogenic tumours and this may be due to low expression of MHC class I (Li *et al.*, 1998). The typical dose used is 1×10^5 cells /mouse, which is 1.5 to 2 times the minimal tumorigenic dose in normal C57BL/6 mice (Overwijk and Restifo, 2001). The cells were cultured in phenol free Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Ham's) (1:1) (DMEM: F12), 2mM L-glutamine and 15mM HEPES 4-(2-hydroxyethyl)-1-piperasineethanesulfonic acid) (GIBCO, 31330). The medium was supplemented with 10% fetal calf serum (FCS) (Life Technologies, Ltd), and 5ml penicillin (100 IU/ ml) /streptomycin (100 µg/ml) (Sigma, P4333). The cells were incubated at 37^0C in a humidified atmosphere with 5% CO_2 and the cells were sub-

cultured twice a week (0.25 % trypsin and 2mM EDTA solution (Sigma- Aldrich, MO, USA, T4049). These cells are able to cause black discoloration of the medium due to the production of melanin pigment (**Figure 2-1**).

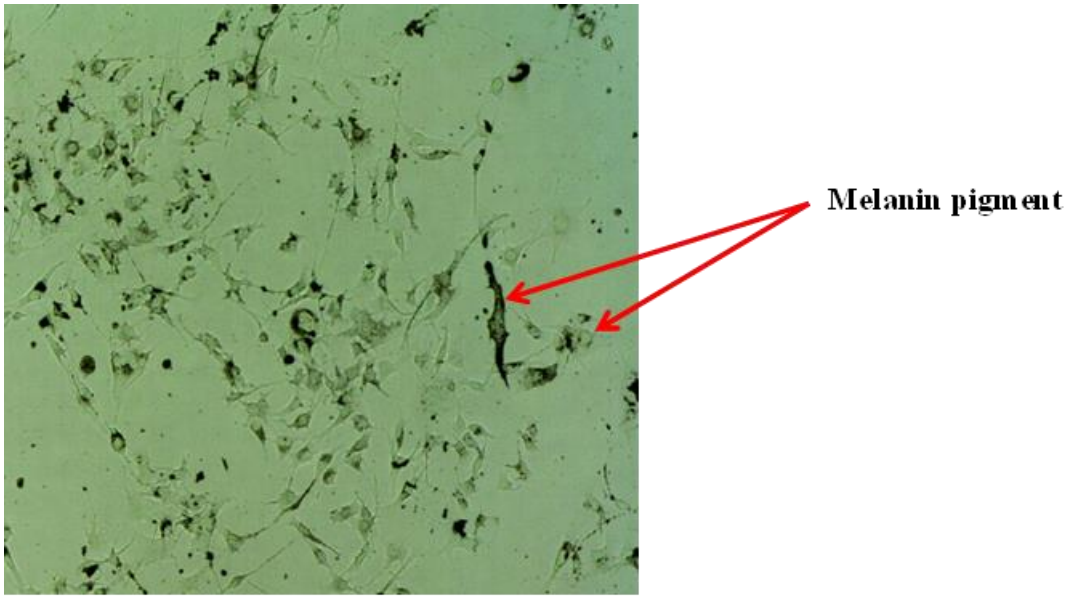


Figure 2-1: Microscopic picture showing murine melanoma cell line (B16-F10).
Cancerous cells, spindle in shape and their confluence about 95 % after 24 hours (40x magnification).

2.3.2 Murine macrophages (J774.2) cell line

The macrophage- like cell line J774.2 is an ideal model for immunological studies, and biophysical analyses of cellular processes (Lam *et al.*, 2009). They were re-cloned from the original ascites and solid mouse tumour J774.1. Therefore, this project used these macrophages to identify the changes in their activity and polarisation from M1 to M2 phenotype. These cells are adherent and they were maintained in RPMI (Roswell Park Memorial Institute medium) (Gibco) supplemented with 10% (v/v) FCS (Life Technologes, Ltd), 2mM L-glutamine, and 5ml penicillin (100 IU/ ml) /streptomycin (100 µg/ ml) (Sigma, P4333),and incubated in a humidified incubator (5% CO_2) at 37 ^{0}C. J774 are firmly attached to the surface therefore a cell scraper was used to gently dislodge cells from the flask and they were sub-cultured twice a week (at 70-80% confluence) (**Figure 2-2**).

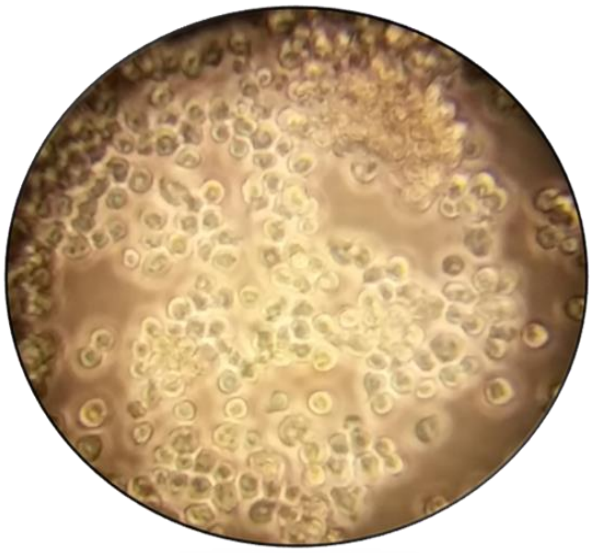


Figure 2-2: Microscopic picture showing murine macrophage- like cell line (J774).
Oval/round and spindle in shape, semi- adherent cells (40x magnifications).

2.3.3 Cryothawing of cells

Cryogenic vials were snap warmed in a water bath at 37^0C and the cells were transferred to a falcon tube containing 10 ml of complete media for washing stage. After that, the tube was centrifuged at 270xg for 5 to 7 minutes, and supernatant was removed. The cells were re-suspended and dispensed into the labelled flasks and incubated at 37^0C in 5% CO_2 air atmosphere for further incubation.

2.3.4 Cryopreservation of cells

Cultures free of any microbial contamination and in log growth phase, pre- confluent in culture were used to prepare stocks of murine melanoma cell line (B16-F10) and murine macrophages (J774) using a slow freezing method. The medium was replaced 24 hours before freezing, the next day the culture media was removed, and discarded from the flask before washing with PBS to remove residual medium. PBS was removed and 0.2 % Trypsin –2mM EDTA solution was added to detach the cells, and the cells were incubated at 37^0C for 5 minutes. Then complete medium was added to inactivate trypsin and, cells were transferred to falcon tube. The cells were counted, centrifuged at 125xg for 5 to 7 minutes before discarding the supernatant. The pellet was re-suspended in 1mL of 10% (v/v) of DMSO/ FCS at a density $1\text{-}5\times10^6$. The culture was put in Cryogenic vial and kept in $^-80\ ^0$C.

2.3.5 Routine Cell culture

All media preparation and all cell culture work were done in class 2 laminar flow hood. Cells were grown as adherent cultures and were maintained in complete medium, and incubated at 37^0C in a humidified atmosphere with 5% $C0_2$. The medium was renewed twice a week during 2-5 days, the cells were grown confluent, start building monolayer and, sub-cultured. The cells were washed with 5 ml PBS twice to remove cell debris and, medium remnants and trypsinised with 3-5ml (according to the size of flask) of 1x trypsin/EDTA (Sigma) diluted in phosphate buffer saline (PBS), to detach the cells, and incubated for 5 minutes. In order to de inactivate trypsin, 4 ml of complete medium was added and, the cells were centrifuged at 125xg for 5 to 7 minutes. The supernatant was removed and the pelts were re suspended in fresh medium. The cells were transferred to new flasks 25 and 75 cm^2 (Nunclon). Murine melanoma cell line (B16-F10) has the ability to produce melanin pigments in culture, which causes black discoloration of their medium. Macrophages cell line differentiated after 3 days and change their shape from oval to spindle shape.

2.4 Cell counting and viability

This test determined the percentage of viable cells in cell suspension. This was done by mixing 10 µl of cell suspension with an equal volume of trypan blue. Then 10 µl of the mixture was loaded into haemocytometer chamber and the total number of cells and the percentage of viable cells were determined.by as following:

Number of cells /ml = number of cells counted per mm^2 × 2 diluted factor $\times 10^4$

Viability of cell (%) = number of living cells /total number of cells ×100

2.5 Preparation of B16-F10 cell conditioned medium

When cells reached about 70% confluence, the conditioned medium was collected and centrifuged at 350xg for 5 minutes. The supernatant was filtered through a 0.2µm filter (PALL) before being used.

2.6 Preparation of cell lysates for Western plots

Murine melanoma cell line (B16-F10) was cultured in complete medium, the supernatants were removed and cells washed twice with PBS to remove any debris, and fetal calf serum. The attached cells were collected in fresh Eppendorf tubes by lysing them in 1ml lysis buffer (**Table 2-2**). The samples were incubated on ice for 30 minutes, centrifuged at 400xg for 5 minutes and supernatants were transferred into new fresh reaction tubes before frozen at $^{-}80$. When required the cells were thawed and subsequently used.

Table 2-2: Preparation of 5ml Lysis buffer

Stock solution	Amount	Source
1M Tris-HCl (PH 7.5)	250µl	50mM
100mM Na Orthovanadate	50µl	1mM
1M Benzamidine	5µl	1mM
100mM PMSF	10µl	0.2mM
Protease Inhibitor Cocktail (Sigma, P830)	20µl	N/A
Beta-Mercaptoethanol	5µl	0.1%
10% Triton X-100	500µl	1%
500mM Na Fluoride	500µl	50mM
MilliQ water	2.81ml	xxxx

*Lysis buffer usually prepared in ice

2.7 Preparation of tissue lysates for Western plots

Soluble proteins were isolated from tumour, liver, colon, brain and spleen using FastPrep-24™ 5G (MP Biomedical, CA, USA) a glass Teflon homogeniser. The tissues were mixed with lysis buffer **(Table 2-2)**, at a ratio of 9 µl /mg of tissues. The mixture was processed by the homogeniser for 10 minutes and was kept on ice for 30 minutes. Subsequently, the mixture was transferred into fresh 1.5 ml reaction tubes and, centrifuged at 1200xg at 4^0C for 10 minutes (Genfuge 24D). The supernatant was transferred to reaction tubes to be frozen at $^-80\ ^0$C.

2.8 Protein Assay (Pierce™ 660nm protein assay)

The protein concentration of the lysates was measured by Pierce 660 nm protein (Pierce™ 660nm Protein Assay Reagent, Cat No. 22660), according to the manufacturer's protocol. Standard curve was prepared by using different amounts (2000, 1500, 1000,750,500, 250, 125, 50 and 0 µg/ml of Bovine Serum Albumin BSA) (PAA, K45-001) dissolved in PBS. Then 10 µl from each dilution of BSA in PBS were put in 96 wells plate (Nunc Maxisorp) in triplicate and 10µl of protein lysates were added in triplicate. Subsequently, 150 µl of the protein assay reagent were added in each well and incubated for 5 minutes at room temperature until colour change. (The change in colour is proportional to protein concentration). Finally, the plate was read spectrophotometrically (TECAN Magellan for F50) at 660nm. The standard curve is made by adding standard reading against optical density and this curve will use to measure protein concentration.

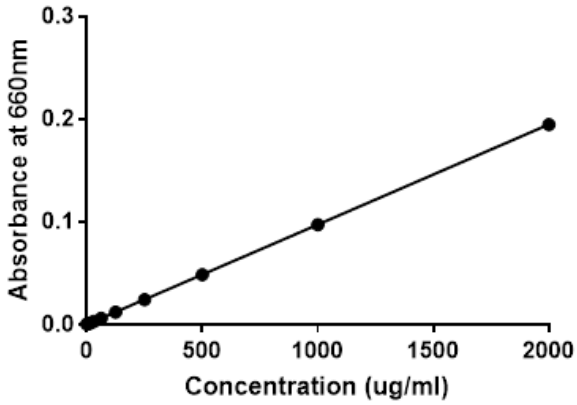


Figure 2-3: Example of a standard curve showing the absorbance of different concentrations of BSA by Pierce 660nm Protein assay.

2.9 Polyacrylamide gel electrophoresis and immunoblotting (Western blot)

2.9.1 Polyacrylamide Gel electrophoresis

Both resolving and stacking gels were prepared according to laboratory protocols. The resolving gel **(Table 2-3)**, was poured between two glass plates until polymerised a stacking gel **(Table 2-4)**, was added. The percentage of the resolving gel depended on the molecular weight of the protein of interest.

The samples were mixed with 2X Laemmli-10mM DTT sample buffer (under reducing condition) **(Table 2-5)**, and heated in dry heat block at 95^0C for 3-4 minutes in order to denature proteins. The Samples were centrifuged in 12,000xg for 10 second and the supernatants were used. Subsequently, 20 µg of total protein was loaded into the wells and 10 µl of pre stained protein ladder (Bio-Rad) was used to assess the relative molecular weight of each protein. The gel was immersed in 10X SDS-Tris-Glycine running buffer (**Table 2-6**), and typically run at 90V for 100 minutes.

Table 2-3: Reagents and volumes used to prepare 10 ml of 10% resolving gel

Solution	Amount	Source
H2O	4 ml	
30% Acrylamide	3.3 ml	(Sigma)
10% SDS	0.1ml	(Sigma)
1.5 M Tris pH 8.8	2.5 ml	
10% (w/v) Ammonium persulfate (APS)	0.1 ml	(Sigma A- 3678)
TEMED	0.006 ml	(Sigma- 9281).

Table 2-4: Reagents and volumes used to prepare 5ml of 5% stacking buffer

Solution	Amount	Source
H_2O	3.4 ml	
30% Acrylamide	0.83 ml	(Sigma)
10% SDS	0.05ml	(Sigma)
1M Tris pH 6.8	0.63 ml	
10% (w/v) Ammonium persulfate APS	0.05ml	(Sigma A- 3678)
TEMED	0.005 ml	(Sigma- 9281)

Table 2-5: Preparation 10ml of 2x Laemmli sample buffer (loading buffer)

Solution	Amount	Source
H_2O	2.8 ml	
1 M Tris-Cl (pH 6.8)	1.2 ml	(Sigma)
Glycerol	2ml	(Sigma)
10% Sodium dodecyl sulfate (SDS) (w/v) in H_2O	4 ml	(Fisher)
Bromophenol blue was added to a final concentration of 0.02% (w/v) and the 2X Laemmli sample buffer was stored at room temperature		
200 mM Dithiothreitol * (DTT), Buffer should be prepared fresh)		(Sigma)

* DTT were not added in case of non-reducing samples

Table 2-6: Reagents and quantities used to prepare 10x running buffer

Solution	Preparation
10x Running buffer	30g Tris-Base (Fisher), 144 Glycine (Fisher) and 10 g SDS in 1L dH_2O (for 1x Running buffer diluted 1:10 in dH_2O).

2.9.2 Coommassie brilliant blue R-250 staining

This stain was used to identify separated proteins in the gel by immersing the gel in Coommassie brilliant blue R-250 stain (3g stain in 100ml acetic acid, 450ml IMS, 450ml H2O), The gel was destined using de-staining solution (50ml IMS,100ml acetic acid, 850 ml H_2O).

2.9.3 Electrophoretic transfer of proteins:

After running SDS-PAGE, gels were placed onto a polyvinylidene difluoride membrane (PVDF) activated with 100 % methanol (GF health care Life science, Amersham Tm Hybond Tm 0.2 µm PVDF, 106006) or Nitrocellulose membrane without activating with methanol. This was placed onto a small stack of Whatman Ltd paper and sponge soaked in 1X transfer buffer (**Table 2-7**). They were arranged in way that allows protein to transfer from gel to membrane (gel-faced cathode (black), while the membrane faced anode (red). Blotting was done either at 100V at using ice pack or overnight at 30V at 4 ^{0}C depending on the experimental conditions.

Table 2-7: Preparation of 1x of transfer (blotting) buffer

Solution	Preparation
1x blotting Buffer	5.9g Tris–Base (Fisher Chemical), 2.9 g Glycine (Fisher Chemical), complete to 1L H_2O. 10 ml of 100% Methanol were added to this solution. This solution should be prepared fresh.

2.9.4 Ponceau S staining

The membrane was immersed in reversible Ponceau S stain (40 ml dH2O, 300µl Glacial acetic acid (Fisher Chemical), 300mg Ponceau S) for 5- 10 minutes to visualise protein transfer. The blot was washed three times in PBS until the stain was fully removed.

2.9.5 Blocking and antibodies incubation

After blotting, the equipment was disassembled and the blot placed in 5 % dried skimmed milk in PBS (w/v) for 2 hours on the shaker. Then the blot was placed in 5% (w/v) dried skimmed milk in PBS containing primary antibody for 2 hours or overnight at 4^{0}C (**Table 2-8**). After washing 3 times 15 minutes each by washing step (with TBS buffer containing 0.05% Tween 20), the blot was incubated in horseradish peroxidase (HRP)-conjugated secondary antibody (**Table 2-9**), diluted in 5% (w/v) dried skimmed milk (Fisher Chemicals) in PBS for 1 hour before being washed 3x in PBS. Normalisation of target proteins was performed against endogenous loading control (β-actin).

Table 2-8: Primary antibodies

Primary antibodies	Dilution	Source
Rabbit polyclonal–anti leptin receptor IgG	1:1000	Santa Cruz Biotechnology ,INC (SC-8325)
Mouse monoclonal anti–β- actin	1:5000	Sigma A(5316)

Table 2-9: Secondary antibodies

Conjugated HRP	Dilution	Source
Swine anti-rabbit polyclonal HRP	1:3000	Dako REF P0399
Goat anti-mouse IgG antibody HRP	1:5000	Biorbyt orb 233570

2.9.6 Visualisation of antibody reactive proteins

2ml of Enhanced– chemi luminescence ECL (Pierce^{TM} ECL western Blotting Substrate, 32106) reagent a mixture of reagent (reagent A or luminal and reagent B) was poured over the membrane for 3-5 minutes. The membrane was coved with cling film and exposed to film (Bio- Max Light, sigma Aldrich, MO, USA) in a light – tight cassette until antibody-reactive bands were detected (exposure: 10-30 minutes). The film was immersed in developing solution for 2 minutes until bands appeared and in water and flooded in a Fixer solution, washed with tap water and left to dry. A UV Transilluminator (BIO-RAD) was used to visualise the bands, which save time and gave more reliable and sensitive detection.

2.10 Mouse Chemokine Array

Samples (cell lysates or supernatant) were transferred to a protein array, which is specifically designed to detect mouse chemokines (Mouse Chemokine Array Kit, R&D systems, Cat no. ARY020).

The Mouse Chemokine Array kit had four nitrocellulose membranes, each composed of 25 different capture antibodies for the 25 different chemokine proteins in duplicate. In order to block the membranes 2ml of blocking buffer was added (provided with the kit) for 1hour at room temperature with shaking. Each sample was mixed with 15µl of reconstitution detection antibody cocktail and incubated for 1hours at room temperature after that the blocking buffer was aspirated from the membranes, and the samples were added to each membrane and incubated overnight at 4˚C on the shaker. The next day the membranes were washed with the washing buffer 3times for 10 minutes, and 2ml of Streptavidin-HRP was added to each membrane and incubated for 30 minutes at room temperature on a rocking platform shaker before being washed as described previously. In order to remove the excess buffer, the edge of each membrane was blotted on the paper towel and after that, the membranes were placed on plastic sheet protector. The face of the membrane with identification number were exposed to Chemi Reagent Mix, composed of 1ml Chemi Reagent A and 1ml of B reagent, covered in cling film and any air bubbles were removed carefully. The membrane was exposed to X- ray film for 2 minutes in a light-tight cassette, the film was immersed in Developer solution for 2 minutes until antibody-reactive spots present, washed in water, and submerged in fixative solution, washed in water and left to dry.

2.11 Proteomic analysis of Mouse XL cytokine array

The changes in cytokine production was evaluated in sera of 6 months age *LDLR*$^{-/-}$ females mice fed on HFD and control diet using Mouse XL cytokine array, the protocol was done as follow:

All reagents were prepared prior to use, 2ml of buffer 6 (block buffer) was added into each well of the 4-well Multi-dish. Membranes were placed in separated wells and the number of these membranes was faced upwards. After one hour incubation on a rocking platform shaker, Array Buffer 6 was aspirated from the wells of the 4-well before preparation of samples by adding up to 180µl of each serum samples to 0.5ml of Array

Buffer 4 in separate tubes, sample volumes were adjusted to a final volume of 1.5 ml with Array Buffer 6. Followed by incubation overnight on a rocking platform shaker at 2-8^{0}C for overnight. Next day, each membrane was washed with 20ml of 1x wash buffer for 10 minutes on a rocking platform shaker (2 times for 3 washes). The membranes were removed from wash container before being incubated for 1 hour in wells contained diluted detection antibody cocktail on a rocking platform shaker. The membranes were washed as described previously and returned into the 4-well multi-dish containing 2ml of 1X Streptavidin-HRP followed by incubation for 30 minutes at room temperature. The membranes were washed and drained, before adding 1ml of freshly prepared Chemi Reagent Mix into each membrane and incubated for 1 minute at room temperature. The membranes were covered with plastic sheet protector and photos were taken by UV Transilluminator (BIO-RAD).

2.12 Cells viability assays

2.12.1 MTS assay

MTS Assay Kit (Cell Proliferation Cat. No. ab197010) was used to measure the mitochondrial cell viability of cancer cells (B16-F10). The cells were plated at a density of 1×10^3 cells/well in a 96 well plate (Nunc Maxisorp) and they were left overnight at 37 ^{0}C to allow cells to adhere. The next day the growth medium was replaced by phenol free DMEM with deferent treatments and concentrations according to experimental requirements. Then, 20 µl of MTS solution (5mg/mL) in DMEM without phenol red (w/v) was added and further incubated for 4 hours at 37^{0}C. MTS was reduced by metabolically viable cells and the density of final yellowish colour was measured spectrophotometrically at 495 nm (TECAN Magellan for F50).

2.12.2 Crystal Violet assay

Cells were plated in 96 well plates (Nunc Maxisorp) at a density 1×10^3 cells/ well, and they were incubated overnight at 37 ^{0}C in humidified atmosphere under 5% CO_2 to allow the cells to adhere. The next day the cells were treated with different treatments as per the specific experimental protocol. The supernatants were removed and the cells were stained with 0.5% filtered crystal violet 50 µl /well, which resolved in 20% (v/v) methanol in H_2O, for 3-5 minutes at room temperatures. The dye was discarded and the

plate was washed 3x under tap water and dried by inverting the plate to take out any additional water and blotted on a paper towel. Then 100 µl of 20% (v/v) acetic acid in H_2O was added in each well to solubilise the accumulated dye in the cell's nucleus and absorbance measured spectrophotometric ally at 540 nm using (TECAN Magellan for F50).

2.13 Cell culture wound migration assay

2.13.1 Protocol

The cells were cultured in the six-well plates at a density of 1×10^6 and grown to confluence for 24 or 48 hours. Optimisation was done to remove poly D-lysine from plate which was found to be unsuitable for B16-F10 and J774. A scratch was made through the cell monolayer by using p200 pipet tips and the media and cell debris were aspirated carefully. The cells were washed slowly twice with 2ml PBS or serum free media to discard fluting cells and to obtain clean wound. Then, cells were left with (2% FCS containing media for B16-10 and 5 % FCS for J774) for 1-2 hours to rest before treating them with different treatments as per the specific experimental protocol. Following the generation and inspection of the wound an initial picture was taken by (Seiss Axiovert 200m fluorescence microscopy/ phase, GERMANY) at zero and 24 hours, Image J was used to analyse the pictures and calculate the differences in the wound healing/cell migration between the treatment and control by flowing this formula:

% of wound healing = (area of wound at time zero - area of wound at time (n) / area of wound at time zero) ×100.

2.13.2 Detection of specific area

In order to obtain the same field during the image acquisition at time zero and next time point, two approaches were used as following:

1- Drawing Vertical and horizontal lines on the back of the plate using ballpoint pen the distance between each lines about 2mm (**Figure 2-4A**).

2- Capturing the images by putting the wound X and Y position of the microscope, the microscope should contain a graded stage in order to use the same positions at different time points (**Figure 2-4B**).

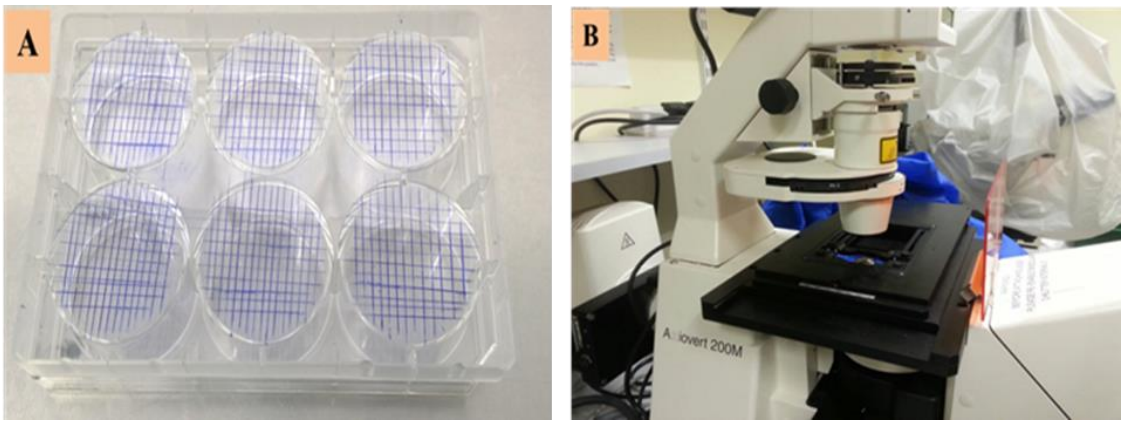


Figure 2-4: strategy used to detect specific areas in six wells plate

(**A**) Drawing 2mm vertical and horizontal lines on the back of the 6 wells plate. (**B**) Zeiss Axiovert 200m fluorescence microscopy/ phase contains a graded stage.

2.14 Long term survival assay

B16-F10 cells were plated at a density of 1×10^4 cells/well in 6-well plates. The next day, the medium was replaced with medium containing 2% FCS and the cells were treated with different treatments as per the specific experimental protocol. Cells were allowed to grow for 10 days with medium changed every 3-4 days. Thereafter, cells were stained with 0.5% filtered crystal violet for 5-10 minutes at room temperature (as described previously). Accumulated dye in the cell nucleus and absorbance measured spectrophotometrically at 540 nm using (TECAN Magellan for F50). The cell survival was quantified using Image J software.

2.15 Enzyme Linked Immunosorbent Assay (ELISA)

This assay was used to quantify the amount of various cytokines in sera of *LDLR*$^{-/-}$ tumour bearing mice fed on different diets as well as supernatants of (B16-F10 and J774 under different conditions (**Table 2-10**), according to following protocol:

ELISA plate (Nunc Maxisorp) was coated with 100µl/well of capture antibody (11µg of antigen –affinity purified Rabbit Anti X + 0.5mg D-mannitol) in 110 µl H_2O diluted in diluent buffer (0.05% Tween-20, 0.1% BSA in PBS) to final concentration of 0.50µg/ml. The plate was sealed and incubated overnight at RT. The next day, the wells were aspirated and washed 4 times using 300µl of wash buffer (0.05% (v/v) Tween-20 in PBS) per well. After the last wash, the plate was blotted on absorbent paper to remove the remaining buffer. Each well was blocked with 300 µl of the blocking buffer 1% (w/v) Bovine Serum Albuminutes (BSA in PBS), followed by incubating for at least 1 hour at room temperature. After that the plate was aspirated, and washed 4 times, 100ul of samples and standard diluted starting from 3ng/ml to zero were added into pre-designated wells in triplicate followed by incubated at for at least 2 hours. The wells were washed 4 times as previously described before adding 100µl of detection antibody diluted in diluent buffer to final concentration of 0.25µg/ml. Then incubated at for 2 hours followed by washing 4 times using wash buffer. 100µl of Streptavidin –HRP conjugate diluted to a concentration of 0.025µg /ml was added per well and incubated 30 minutes at room temperature. The wells were aspirated and washed by washing buffer before adding 100µl/well of 1xTMB peroxidase ELISA substrate kit (BIO-RAD Cat: 172-1068). Incubation for 20 minutes for colour development, 100ul of 1M HCL stop solution was added to each well and the optical density at 450 nm, with correction at 680 nm was set up using an ELISA plate reader (LT-4500, labtech). The optical density values were corrected to the blank and the X in supernatant samples was determined through the linear equation of the standard curve.

Table 2-10: Cytokines and Chemokines that were investigated in this study by ELISA

Samples	Chemokine/cytokines	Cat number	Company
High fat diet (58R3) control diet (58R1) 58R3 + VitD3 Supernatants of B16-F10 Supernatants of J774	TNF-α	Cat # 900-TM54	PEPROTECH
High fat diet (58R3) control diet (58R1) 58R3 + VitD3 Supernatants of B16-F10 Supernatants of J774	CCL2	Cat # 900-M126	PEPROTECH
High fat diet (58R3) control diet (58R1) 58R3 + VitD3	IL-6 Leptin IL-10	Cat # 900-TM50 Cat # 900-K76 Cat # 900-TM53	PEPROTECH

2.16 Determination of Non-Esterified Fatty Acids (NEFA)

The Wako NEFA C test kit (999-75406-01) GB- 0207 D4 was used as an *in vitro* enzymatic colorimetric method for the quantitation of non-esterified fatty acids (NEFA) in serum.

7ul of serum, and NEFA standard diluted with PBS were added into 96 well/plate flat bottom in triplicate. 200µl of colour reagent A prepared by dissolving the contents of R1a (colour reagent A), with 10 mL of R1(solvent A), and mixed well according to manufacturer's instruction to each well followed by incubation 37°C for 5 minutes. After that, the absorbance of each well was measured at 550nm (Abs1) (sample blank). 100µl of colour reagent B solution prepared by dissolving the contents of a bottle R2a

(Color reagent B) with 20 mL R2 (Solvent B) before adding into each well, the plate was incubated at 37^0C for 5 minutes, and measured at 550nm by using ELISA plate reader (LT-4500, labtech, UK). First reading was subtraction from the second reading in order to obtain the results, absorbance versus concentration was plotted to construct the calibration curve.

2.17 Hematoxylin and eosin staining

Formalin-fixed paraffin-embedded (FFPE) tumour tissues were cut into 4- μm thick sections, and the sections were de-paraffinzed through 100% xylene for 5 minutes, hydrated in a graded series of industrial methylated spirit (IMS), 100% IMS and 75% IMS for 5 minutes each. The sections were washed with running tap water for 10 minutes, stained with Mayer's Hematoxylin for 5 minutes and washed with tap water for 5 minutes. The section were immersed in acid alcohol for few seconds, stained with Eosin stain 1% Eosin in H_2O (w/v) for 2-3 minutes before being washed with running tap water and distilled water for 5 minutes. The sections were cleared through 100% Xylene for 2 minutes and mounted in DPX (Dibutyl Phthalate Xylene) solution. The sections were left at room temperature for 24 hours before they were evaluated using a light microscope (OLYMPUS connected to camera (INFENITY).

2.18 Oil red O staining

Oil red O stain was prepared 0.1% oil red O in Isopropanol (w/v), and the stain was pre-heated in a water bath (Electro thermal) for 30-60 minutes at 56 ^{0}C, and to cool for 1 hour at room temperature. 30 ml of stock solution was diluted with 20 ml of distilled water before filtering through 0.2 μm filter (Pall Corporation).

2.18.1 Protocol for staining cells

Lipid inclusion was measured in the murine melanoma cell line (B16-F10) that were cultured in six-well plates with sterilized glass coverslip at a density of 1×10^5 cells /well and cultured for 24 hours. The medium was replaced with different treatments as per the specific experimental protocol. The cells were rinsed with PBS three times for 2 minutes each and fixed with 10% paraformaldehyde for 10 minutes at room temperature. The cells were treated with fresh filtered oil red O solution for 10 minutes.

After washing the cells, slides were counterstained with haematoxylin for 2 minutes. Intracellular lipid droplets were observed and captured with a light microscope.

2.18.2 Oil Red O staining measurement by spectrophotometer

The cells were washed three times with ice cold PBS for 2 minutes and fixed with 4% formaldehyde for 10 minutes. The cells were washed 3x with PBS before staining with Oil Red O solution for 10 minutes at room temperature. In order to remove unbound staining, the cells were washed with PBS three times for 2 minutes. Dimethyl sulfoxide (DMSO) was added to each sample with shaking at room temperature for 5 minutes. The samples were transferred to a macro cuvette and the optical density was measured by spectrophotometer at 510 (JENWAY, 6715 UV/VIS).

2.19 *In vivo* model of Melanoma tumour in *LDLR*$^{-/-}$

2.19.1 Establishment of melanoma tumour Model

2.19.1.1 Preparation of Murine melanoma cells (B16-F10)

B16-F10 cells were maintained in phenolphthalein free ATCC- formulated Dulbecco's modified Eagles medium (DMEM/F-12) with L- Glutamine and 15mM HEPES (gibco /11039-021).This medium was supplemented with 10% fetal calf serum (FCS), and 100 IU/ml penicillin and 100µg/ml streptomycin). It was found that phenol red has estrogenic activity and when added to the culture medium promotes cell proliferation and cell cycle progression (Węsierska-Gądek *et al.*, 2007). Therefore, in order to avoid any interference with results, phenolphthalein was not be used in this study.

2.19.1.2 Protocol

On the morning of the day of injection, the flasks were washed twice in PBS to remove any floating cells and residual medium. 2-5ml of (0.2 % Trypsin –2mM EDTA solution (Sigma- Aldrich / T4049), was added to detach the cells. The cells were incubated at 37 ^{0}C with 5% CO_2 for 5-10 minutes before adding complete medium to inactivate trypsin. The cells were transferred to a centrifugation tube and spun at 125xg for 5 to 7 minutes. The supernatant was then discarded and the pellet was washed three times in PBS. The adherent cells were counted using a haemocytometer and the viability checked by using trypan blue exclusion. The experiment with these cells were conducted within three passages of thawed cell stocks and they were used when they were in logarithmic

growth phase (≤ 50% confluent in 75 cm^2 tissue culture flask) and more than 90% of cells were viable.

2.19.2 Subcutaneous syngeneic tumour implantation

In order to establish the model in mice fed on a high fat diet and estimate the length of time of permissible tumour growth, *in vivo* experiments were conducted to induce tumour implantation using a murine melanoma cell line (B16-F10) as a model of tumour growth in *LDLR* deficient mice (as a model of familial hyperlipidemia).

A suspension of B16-F10 mouse melanoma cells (5×10^5 cells in 100µl PBS) (Overwijk and Restifo, 2001), were subcutaneously injected into the right rear flanks of *LDLR* $^{-/-}$ mice using Insulin syringe (29 G ½) in (0.5 ml, Terumo) (supervisor did this part of work). The injected cells were allowed to proliferate and establish themselves as solid tumour between 10-14 days, once the tumors became palpable after this period mice were culled by cervical dislocation. Mice were weighed weekly to determine the changes in the body weight and tumour development (**Figure 2-5**).

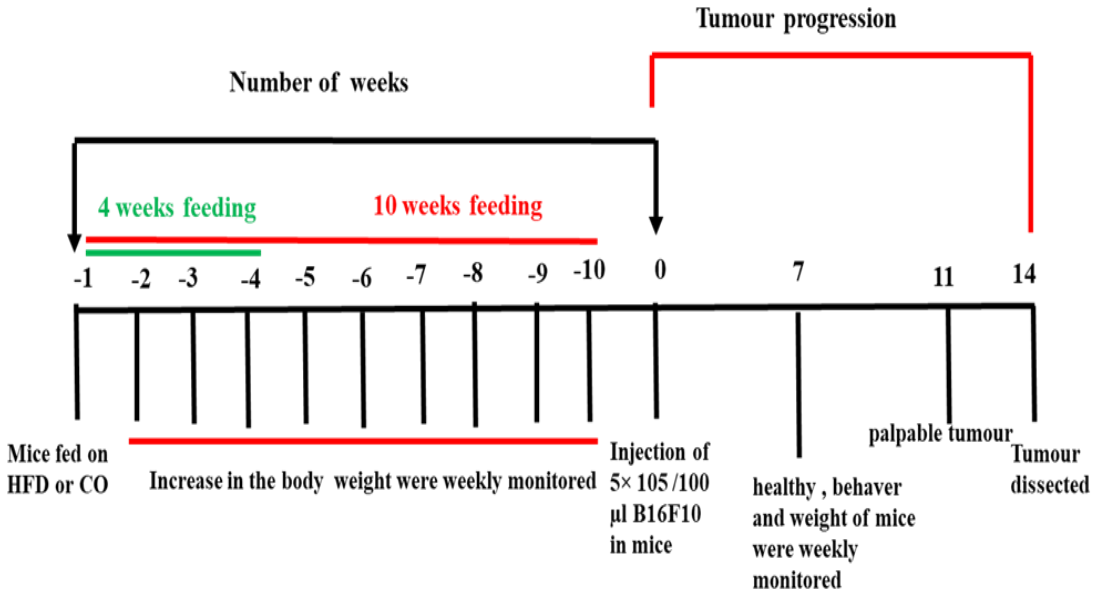


Figure 2-5: Experimental strategy used to study the impact of diet-induced obesity on melanoma progression in HFD *LDLR*$^{-/-}$ mice with a C57BL/6J background.

2.19.3 Organ harvest from mice

At the endpoint, typically 10-14 days post-injection, mice were euthanised by isoflurane overdose and cervical dislocation. Mice were weighed, dissected and examined for internal metastases. Tumour size was measured by using dial calipers and tumour weight was recorded and divided into two parts; one was formalin fixed and the other half-snap frozen at $^{-}80^{0}$C for further characterisation. Additionally, blood samples were collected in Eppendorf tube and centrifuged at 400xg for 10 minutes and subsequently, serum was collected and frozen at $^{-}80^{0}$C.

2.20 Extraction of Ribonucleic Acids (RNA)

2.20.1 RNA extractions from tissues

The organs of interest (brain, liver, colon and tumour) were placed into a tube containing an appropriate amount of TRI Reagent (Sigma-Aldrich) (1 ml per 100 mg of tissue), and homogenised using a glass Teflon homogeniser.

2.20.2 RNA Isolation from cell lines

The medium was removed from 25 and 75 cm^2 tissue culture flasks containing cells at 80-100% confluence before adding 1 -3 ml of Trizol reagent (Sigma-ALDRICH, UK). The cells were detached using a scraper, and collected into reaction tubes. Thermo SCIENTIFIC (Cat. No. #K1632) kit was used to extract RNA according to manufacturer's instructions:

The samples were left to stand at room temperature for 5 minutes. After adding 200 µl of chloroform (Fisher Chemical) per ml of TRI Reagent used, the samples were shaken for 15 seconds and incubated for 5–15 minutes at room temperature. The samples were centrifuged (GenFuge 24D) at 12000xg for 15 minutes. The colorless upper layer (aqueous phase) which contains RNA was collected into a fresh reaction tube using filter tips. Then, 500 µl of Isopropanol (Fisher Chemical) was added; samples were mixed and left for 24 hours at 4^{0}C to precipitate the RNA prior centrifugation at 12000xg for 8 minutes. The supernatants were removed, and the RNA pellet on the bottom of the tube was washed by adding 1ml of 75% (v/v) ethanol in H_2O. The sample was vortexed (Stuart), and centrifuged at 7,500 g for 5 minutes. Ethanol was discarded, and the pellets were briefly dried for 5–10 minutes. The RNA pellet was resuspended in

25µl of DEPC-treated dH_2O (0.02% (v/v) Diethyl-pyrocarbonate) (Sigma D- 5758). To complete dissolution, the samples were mixed by repeated pipetting for 10–15 minutes at 55–60 ^{0}C using hot plate (Grant).

2.20.3 DNase treatment of RNA samples to remove genomic DNA

In order to remove genomic DNA (gDNA) contamination DNase digestion of Trizol-based Nucleic Acid isolation (Thermo SCIENTIFIC Cat. No. #K1632) was done. In a centrifuged DNase –free reaction tube the following components (**Table 2-11**) were added to each RNA samples to a final volume of 100µl (the process were performed on ice).The concentration of RNA was measured using a Nanodrop spectrophotometer (Thermo scientific) in triplicate (the mean of three readings was taken). The RNA samples were stored at -80^{0}C.

$$\frac{\text{RNA concentration}}{1000} \longrightarrow \frac{\text{RNA con./1000} \times \mu\text{l ?}}{10\ \mu\text{g /ml}}$$

Table 2-11: Reduction of genomic DNA mixing reaction reagents.

Reagent and concentration	Volume
10x DNase I buffer	10 µl
DNase I (2 Units/ µl)	5 µl
RNA (10 µg)	x µl
Incubation for 30 minutes at 37 °C	
EDTA (0.5 M)	1 µl
DEPC H_2O	Up to 100 µl
Incubated for 10 minutes at 65 °C, then kept at -20 °C	

***The volume of the RNA was varied for each sample; however, a concentration of 10 µg was used and complete until final volume with water.**

2.21 Preparation of Complementary Deoxyribonucleic Acid

Thermo scientific Revert Aid H Minus kit (Cat. No. #K1632) was used according to the manufacturer's instructions to synthesise cDNA. The RNA volume needed was calculated by the following equation (RNA quantity =5000g/ total RNA). The following reagents were added to the nuclease free 0.2ml tube (A):3 µg of total RNA, 1µl of Oligo (dT) 18 primer, then the total volume was made up to till 12 µl with water (nuclease free). Tube (B) was prepared by adding 4µl 5x reaction Buffer, 1µl of Ribo Lock RNase Inhibitor (20 U/µl), 2µl of 10mM dNTP Mix, and 1µl of Reverse Transcriptase (200 U/µl). The tube A was run for 5 minutes for initial denaturation at 65.0°C. Tube (B) was added to tube (A) for final volume 20µl. The mixture was incubated for 60 minutes at 42^0C, and for terminating the reaction (inactivation of enzyme) at 70^0C for 10 minutes in the PCR machine (TECHINE,TC-512). The cDNA was stored at – 20^0C until use.

2.22 Reverse Transcriptase Polymerase Chain Reaction (RT- PCR)

The following reagents were mixed in a PCR tube:

10.8 µl of PCR – grade d.H2O, 1µl of cDNA , 2.5 of µl 10X PCR buffer , 4 µl of 1.25 mM dNTP Mix, 2.5 µl of 25 mM MgCl2, 2 µl (5 µM) of forward Primers (100 µM stock) ,2 µl (5 µM) of reverse Primers (100 µM stock), 0.2 µl of Taq DNA polymerase (5 U/ µl) . The total amount of reagent in PCR tube was 25 µl.

The PRC tube was put in the PCR machine (TECHINE, TC-512) (under the following conditions:

Initial denaturation at 94^0C for 2 minutes, Denaturation at 94^0C for 1 minute, Annealing at (53- 66 ^{0}C depending on the primer sequence, for 1 minute, extension at 72^0C for 1 minute , Final extension at 72^0C for 10 minutes and the reaction hold and stabilised at 4^0C and the number of cycles was 30 cycles. The amplicons were analysed by gel agarose electrophoresis (0.7-2%).

2.22.1 Agarose gel electrophoresis

1% agarose in 1x Tris boric acid EDTA (TBE) (89 mM Tris base, 89mM Boric acid, 2 mM EDTA) (w/v), the percentage of agarose is varied according to the molecular weight of gene of interest) was heated by putting the gel in a microwave until the agarose had fully dissolved.

The molten agarose was allowed to cool to approximately 50^0C and Ethidium bromide was added (10µg /ml) (Sigma -Aldrich, Gillingham, UK). Then, 2µl of loading dye (10x DNA gel loading dye, 15% (w/v) BP blue, 50% (v/v) glycerol and 0.5mM EDTA) was added to the PCR product for DNA visualization under the Ultra Violet light. The gel was added into the sealed gel-casting chamber of a proprietary horisontal gel electrophoresis instrument. The sample wells were formed by inserting comb inside the molten agarose. The comb was removed after the gel cooling for approximately 40 minutes and the gel was transferred into an electrophoresis tray. After that the gel was immersed with a suitable volume of 1x TBE. 7µl of each sample and 5µl of GeneRuler) standard site (75bp-1 kb) was run alongside the samples (Invitrogen by life technologies ^{Tm}, Tracklt ^{Tm} 1kb plus DNA Ladder, 10488-085) to determine fragment size. The electrophoresis was performed at a voltage of 90V for 60 minutes; the negatively charged DNA migrates to the positive electrode. The fragments were visualised on an Image quant 100 (UV Trans illuminator) and photographs were taken using an Olympus camera connected with Image Quant 100 capture software (GE Healthcare, UK).

2.23 Real- Time Quantitative polymerase chain reaction (RT-qPCR)

Normalised amount of RNA was used to synthase cDNA and RNase-free DNase kit (Sigma-Aldrich) was used to digest genomic DNA as described previously. cDNA was used as a template for qPCR. The qPCR reactions were performed in a 20 µl total volume, containing a master mix prepared using 10 µl of Sensi Mix TM SYBR Kit (Bioline, London, UK Cat.QT605), and 2µl of (5 µM) of forward, reverse primers of genes of interest (**Table 2-13**), and 3µl of RNase free water (**Table 2-12**). Subsequently, 17 µl of the master mix was added to 3 µl of diluted cDNA (previously diluted 1:4 in RNase free water). A negative control (non-template control) was prepared using water instead of the cDNA template. 20 µl total volume qPCR reaction was run in a Corbett Rotor-Gene ™ 6000 machine and x companies software was used

for relative quantification of gene expression levels and were presented based on comparison to housekeeping gene (GAPDH,β-Actin and β2M mRNA expression). This experiment was conducted in a CAT2 hood in a sterile environment without light to avoid any contamination as well as to prevent SYBR Green degradation. The PCR tubes were loaded in the (Bio-Rad Thermocycler) PCR machine and the temperature cycling conditions were 1 cycle of 95 ^{0}C for 10 second, 40 cycles of 95 ^{0}C for 15second, annealing temperature 60 second, 72 ^{0}C for 15 second, the melting curve program consisted of temperatures between 55^{0}C to 95 ^{0}C. All the samples were run in duplicate and strip tubes and (0.1 ml caps) were used (Qiagen, 981103).

Table 2-12: Preparation of RT-qPCR reaction

Material	Amount
2x Bio Script Bioline SYBR	10 µl
Rnase free water	3 µl
Diluted cDNA (1:4)	3 µl
5 µM Forward primer	2 µl
5 µM Reverse primer	2 µl
Total	20 µl

Table 2-13: Sequences of oligonucleotides that were used in this study

Gene name	Sequence of primers	Size (bp)	Annealing Temp. °C	Reference sequence accession number NCBI
GAPDH	5'-CCTGGAGAAACCTGCCAAGTATG -3' 5'-AGAGTGGGAGTTGCTGTTGAAGTC-3'	130 bp	55	NM-0080848
MCP-1	5'- CACTCACCTGCTGCTACTCATTCAC-3' 5'-GGATTCACAGAGAGCGAAAAATGG-3'	490 bp	57	NM_ 0011333
β2M	5`-GACCGGCCTGTATGCTATCC-3' 5`CAGTAGACGGTCTTGGGCTC-3`	300bP	55	NM_ 0073935
β-Actin	5'-CACCAACTGGGACGACAT-3' 5'- ACAGCCTGGATAGCAACG-3'	55 bp	55	X00351.1
iNOS	5'- TAAAGATAATGGTGAGGGG-3' 5'-GTGCTTCAGTCAGGAGGTT-3'	270 bp	60	NM_ 010927
Arginase-1	5'-AGGAACTGGCTGAAGTGGT-3' 5'- GATGAGAAAGGAAAGTGGC-3'	220 bp	60	NM_ 007482
PGR120	5'-TGTCGCTGTTCAGGAACGAA-3' 5'-CGTAGATGCCTGCTGTTGGA-3'	235bp	60	NM_ 181748

2.24 Data Analysis and calculation of qPCR Using the $2^{\wedge-\Delta\Delta CT}$ Method

The CT values provided from real-time PCR instrumentation are easily imported into a spreadsheet (**Figure 2-6**). CT is defined as the number of each cycle that is required for the fluorescent signal to cross the threshold To demonstrate the analysis, data are reported from a quantitative gene expression experiment. The change in expression of target genes was normalised to the housekeeping gene. Duplicate samples were collected at each time point. Real-time PCR was performed on the corresponding cDNA synthesised from each sample.

To calculate the relative expression of a target gene in each sample, the ΔCT method (Livak and Schmittgen, 2001), and Microsoft office Excel (2001) were used to calculate the normalisation of target genes to housekeeping genes (**Table 2-13**).

All other statistics were performed using Graph pad Prism 7 (Graph pad software).

ΔCT (test) = CT (target gene) - CT (ref gene)

ΔCT (calibrator) = CT (target gene) - CT (ref gene)

ΔΔCT= test ΔCT - calibrator ΔCT

2^-ΔCT= the level of the gene expression

CT: Cycle number at which detectable signal is achieved.

Calibrator: The control sample, meaning an untreated sample.

Test: Test sample means treated.

Reference gene (ref): The reference gene is one that is expressed at a constant level in all test and control samples without being affected by the experimental treatment in the study.

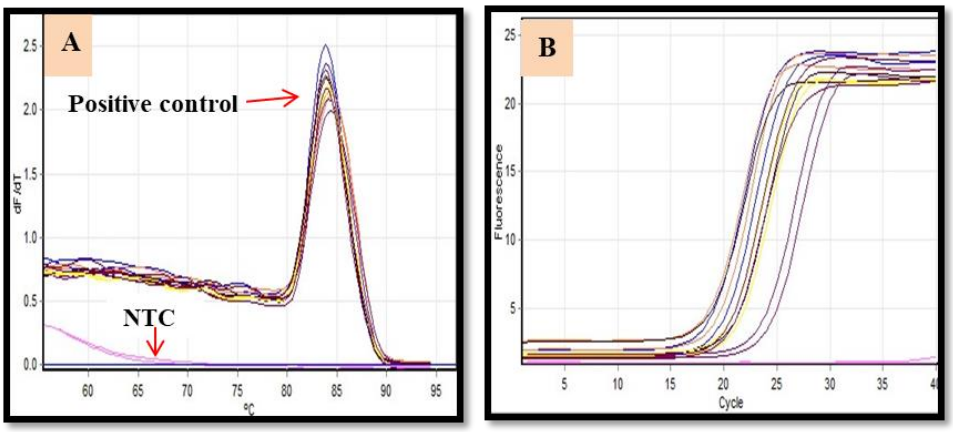


Figure 2-6: Representative melting curve and amplification curves obtained by real-time PCR.

(**A**) The melting curve analysis confirming specific amplification. (**B**) Amplification curves from the qPCR analysis; each assay target is represented by a different colour. Indicate non-template control.

2.25 Flow cytometry

2.25.1 Preparation of single cell suspensions from mouse spleen

Spleens were placed in a Petri dish containing PBS on ice. Sterile forceps were used to place the spleen on a sterile cell strainer mesh (BD falcon 40µM Nylon).This cell strainer was pre-soaked by adding (RPMI-1640, with L-glutamine and sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture, Cat. No. R8758-500ml). The rubber end of the plunger of a 10 ml syringe was used to push the spleen carefully through the cell strainer into a 50 ml falcon tube. The splenocytes were centrifuged at 270xg for 5 minutes at 4^0C and the supernatant was aspirated. Subsequently, the pellets were resuspended in 1 ml of solution (10 % DMSO, 80% FCS, and 10% DMEM v/v). The vials were labelled and kept in -80 ^{0}C for further investigations.

2.25.2 Detection of T-regulatory lymphocytes in mouse spleen (T-regs)

Flow cytometry was used to analyse the percentage of T-regs (as a proportion of CD4 T cells) in the spleen of naïve *LDLR*$^{-/-}$ mice fed on different diets. A T-reg detection kit (CD4/CD25/FOXP3) PE mouse (130-094-165) was used for detection of T-regulatory. Splenocytes were thawed and washed as described previously and the number of splenocytes was counted using haemocytometer. 10µl of trypan blue stain were added to 10µl of splenocytes. 10 µl of stained splenocytes were placed on the haemocytometer to

count the living cells under the light microscope (Nikon Eclipse TS100, magnification 10x). The number of living splenocytes was adjusted to $1x10^6$ cell/100µl in FACS buffer and pre-blocked with Fc receptor specific anti-mouse CD16/32 Antibody (Biolegend, Cat. No. 101302, 2.5 µg/ml). After that, the cells were vortexed, incubated for 15-30 minutes on ice, and covered with foil. The pellets were re-suspended in 1-2 ml buffer to wash away any excess blocking antibody, centrifuge by (BECKMAN COULTER, Allegra ^{TM} X-22) at 300xg for 10 minutes at 4^0C and the supernatant was a spirited completely. Subsequently, the pellets were re suspended with 90 µl of FACS buffer and 10 µl of CD4-FITC and 10 µl of the CD25 antibody were added. The stains were mixed and incubated for 10 minutes in the dark in the refrigerator at (2−8 ^{0}C). Splenocytes were washed by adding 1−2 ml of FACS buffer per $1x10^6$ cells and centrifuged at 300xg for 5 minutes at 4^0C. The supernatant was aspirated before performing the intracellular staining of Anti-FoxP3 antibody.

The pellets were re-suspended with 1 mL of cold, freshly prepared Fixation / permeabilisation solution according to manufacturer's instructions. The stained cells were mixed well and incubated for 30 minutes in the dark (2−8 ^{0}C). Subsequently, splenocytes were washed by adding 1−2 ml of cold buffer and centrifuged at 300xg for 5 minutes at 4°C. The supernatant was aspirated completely before washing the cells by adding 1−2 ml of cold 1× Permeabilisation buffer and centrifuged at 300xg for 5 minutes at 4 ^{0}C. The supernatant was aspirated and the cells were re-suspended in 80 µl of ice-cold 1× Permeabilisation buffer before incubating for 5 minutes in the refrigerator (2−8 ^{0}C). 10µl of the Anti-FoxP3 antibody (company and clone) was added and mixed well followed by incubating for 30 minutes in the dark in the refrigerator (2−8 ^{0}C). The cells were washed by adding 1−2 ml of cold 1× Permeabilisation Buffer and centrifuge at 300xg for 5 minutes at 4 ^{0}C. The supernatant was aspirated and pellets re-suspended with 200µl of 2% paraformaldehyde (fixation buffer) before transferring into Polypropylene tube (Axygen™ Mini Tube System) for analysis by flow cytometry. Acquisition was done using BD FACS Diva ^{TM} software version 8.0 runs on the Microsoft Windows 7 (32-bit) operating system.

2.25.3 Flow cytometry for detection TLR-4

The expression of TLR-4 was investigated in murine melanoma cell line (B16-F10) treated with 450µM Free fatty acids. Tissue culture medium was removed from the flask, and the cells (B16-F10 and J774 was used as positive control) were washed twice with PBS. The cells were detached before centrifugation and the cells were resuspended in an appropriate volume of FACs buffer. Subsequently, the cells were counted and viability analysis was performed. The number of cells was adjusted to 1 x 10^7 cells/mL and FACS buffer (100µl) containing 1x10^6 cell was added to each Eppendorf tube (3 in total for each sample). 0.5 µl of Purified anti-mouse CD16/32 Antibody (Bio legend, concentration 0.5 mg/ml, Cat. No. 101302, diluted 1:200/working concentration 2.5 µg/ml) was added per 100 µl of cells and incubated in dark on ice for 30 minutes. 100µl of FACS buffer was added to each tube to wash the cells before centrifugation at 350xg for 5 minutes at 4°C using (Sigma 1-14 K centrifuge). The supernatant was removed and the pellets were resuspended with 90µl of FACS buffer before adding 10µl of Monoclonal Rat IgG_{2A} PE-conjugated Antibody isotope control (R&D Systems) (tube), 10µl of anti-mTLR4 Monoclonal Rat $IgG2_A$ PE conjugated (FAB275 (R&D Systems) (tube), while the third tubes was left for unstained cells. The cells were incubated for 30 minutes at room temperature in the dark. After incubation, unbound antibody was removed by washing the cells in FACS Buffer. The suspended cells were centrifuge at 300 xg for 5 minutes and the buffer was decanted. 100µl of 2% Paraformaldehyde (to fix the cells) was added to the pellet before transferring into Polypropylene tube (Axygen™ Mini Tube System) for analysis by flow cytometry. The cells were analysed using, BD FACS Diva ^{TM} software version 8.0 runs on the Microsoft Windows 7.

2.26 Statistics and Data analysis

The analysis of replicates was carried out for all experiments in this study. Unpaired T-tests were used to determine if there were differences between the means of two independent groups using Graph Pad Prism 7 (Graph Pad, San Diego California, USA).

Shapiro-Wilk normality test was used to determine whether data were normally distributed. One-way ANOVA with Dunnett's post-hoc tests were used for multiple comparisons when comparing more than two groups. Kruskal-Wallis tests with Dunn's multiple comparisons test were used for non-parametric data. Data were expressed as Means ± SEM for most experiments (n= the total number of mice). Values with a *P*-value less than or equal to the alpha ($P<0.05$) were deemed significant and the null hypothesis was rejected. Statistical significance of results was denoted by either * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

Variation between individuals was minimized as follows: *in vivo* work all mice were kept in the same unit so they exposed to the same stimuli, and littermates were analysed. The ages of mice were matched; the stress was reduced due to environmental enhancement. *In vitro,* cultured according to standard protocols so they were used in comparable growth phases throughout the study. Mycoplasma teat was carried out for all cell lines that used in this study murine macrophages (J774), and melanoma cancer cells (B16-F10) to ensure that all cell lines were free of contaminating with Mycoplasma. This process was repeated in regular intervals as quality control prior to injection of mice. Appropriate controls were used for each stimulation to compare against treatment.

Chapter 3

In vitro influence of fatty acids on the pro- tumorigenic phenotype of co-cultured macrophages and melanoma cells

In vitro influence of fatty acids on the pro- tumorigenic phenotype of co-cultured macrophages and melanoma cells

3.1 Introduction

Adipocytes secrete adipokines and chemokines that enhance tumorigenesis (Makki *et al.*, 2013). The unique and canonical function of adipocytes is to store triglycerides and to secrete fatty acids (FAs). FAs are the basic components of triglycerides (Raynor *et al.*, 2015; Offermanns, 2014; Cullberg *et al.*, 2014). Oleic acid and palmitic acid are the most abundant dietary and plasma FAs. Epidemiological evidence and experimental animals show that diets rich in saturated fat such as Palmitic acid significantly increases the risk of developing insulin resistance, cardiovascular disease and several types of cancer, whereas, consumption of monounsaturated fatty acids like Oleic acids could decrease these risks (Hu *et al.*, 2018). Cancer cells can utilise lipids or fatty acids either from the diet through enhancing *de novo* fatty acid synthesis pathways, or by lipid-transfer from neighbouring adipocytes (Kwan *et al.*, 2014). The enhancement of *de novo* fatty acid synthesis is an important pathway among all the altered metabolic pathways in cancers (Furuya *et al.*, 1997), as its activation has been demonstrated in various types of cancers and inhibition induces apoptosis in these cancers (Kwan *et al.*, 2013). Interestingly, palmitic acid can protect cancer cells from the apoptosis resulting from fatty acid synthase pathway inhibition (Chajes *et al.*, 2006; Pizer *et al.*, 1996). Kwan *et al* (2014) found that malignant melanoma cells that grow in the anatomical vicinity of adipose tissue have higher levels of palmitic acid compared with healthy skin cells and, they utilise the exogenous palmitic acid to fuel cancer cells pathogenicity.

Conversely, the expansion in adipose tissue is markedly associated with increased macrophage infiltration. Macrophages are the main cells in the inflammatory response and also the most abundant cell type in tumour microenvironments (TME) (Bingle *et al.*, 2002; Qian and Pollard, 2010). TAMs facilitate tumour progression by different mechanisms and their presence is considered a sign of poor tumour prognosis (Caso *et al.*, 2010; Rodriguez *et al.*, 2013). Saturated fat induced pro-inflammatory changes in adipose tissue macrophages (ATM) by involving Toll-like receptor-4 (TLR-4) during long term consumption of dietary fat (Shi *et al.*, 2006). Undesirable changes in macrophage activity may influence the growth and development of several cancers

including melanoma (Camell and Smith, 2013). These cells are described as either classically activated, M1-type, which has tumoricidal capabilities or alternatively activated, M2, specialised to suppress inflammation and aid in angiogenesis (Mantovani *et al.*, 2017).

3.2 Aims

The aim of this chapter is to answer important questions that relate to the interaction of two key cells within the complex tumour microenvironment in the presence of a mixture of diet relevant fatty acids (FAs). Murine macrophages (J774) and melanoma cells (B16-F10) are easily grown in culture and were ideal to test the response to monounsaturated oleic and saturated palmitic acids. An important analytical feature is to precondition the cells prior to stimulation with FAs to compare the effect of FAs and secretion factors in conditioned medium of cancers on the tumorigenic phenotype of macrophages and melanoma cancer cells. Therefore, cells will be by analysed for cell viability, migration, chemokine and cytokines secretion and expression of related receptors (GPR120, TLR-4 and Leptin), using MTS , wound healing assay, proteome profile, ELISA, qPCR and flow cytometry, respectively. In some experiments, Bovine serum albumin (BSA) was added to the fatty acids mixture (oleic and palmitic acids). An overview of the experimental design is given in figure (**3-1**).

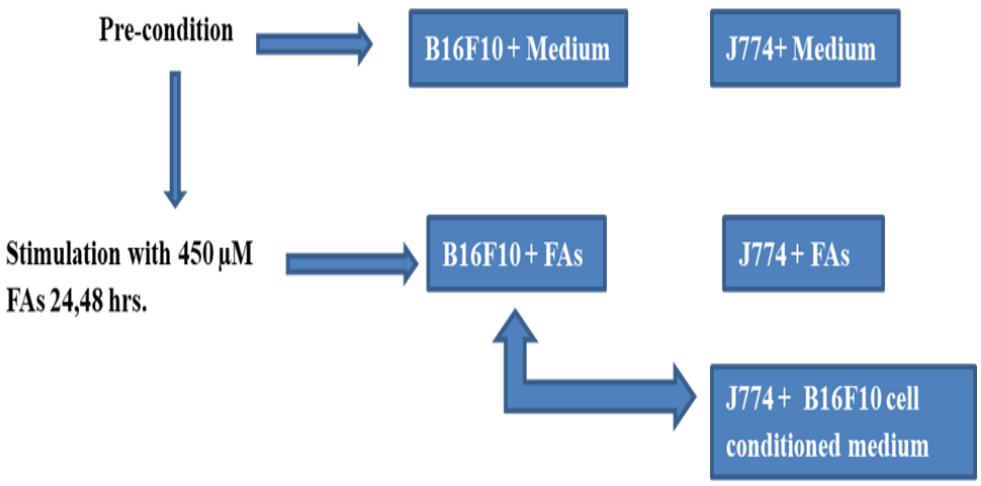


Figure 3-1: Schematic of *in vitro* experimental design.

3.3 Results

3.3.1 Employing Crystal Violet assay for determining cytotoxic effect of FAs on cultured cells

Nucleic acid uptake activity was investigated using a Crystal violet staining method as an indicator of cell viability of the cultured cell lines (B16-F10 and J774). The cell viability of J774 decreased significantly after treatment with higher concentrations of FAs (450 µM) (p=0.0001), compared to both unstimulated cells (control) and 25 and 50µM concentrations (p= 0.7794 and p =0.6805), respectively (**Figure 3-2A**).

This indicates that FAs have a toxic effect on J774 macrophages at 450 µM. In contrast, fatty acids supplemented with 40mg/ml (w/v) BSA had a protective role on macrophage under 450 µM FAs treatment. However, stimulation with 450µM FAs significantly increased the viability of B16-F10 cells (p=0.0253) (**Figure 3-2B**). It is important to note that the concentrations of FAs in FCS and BSA were very low compared with the FAs and supplemented medium (Raynor *et al*., 2015).

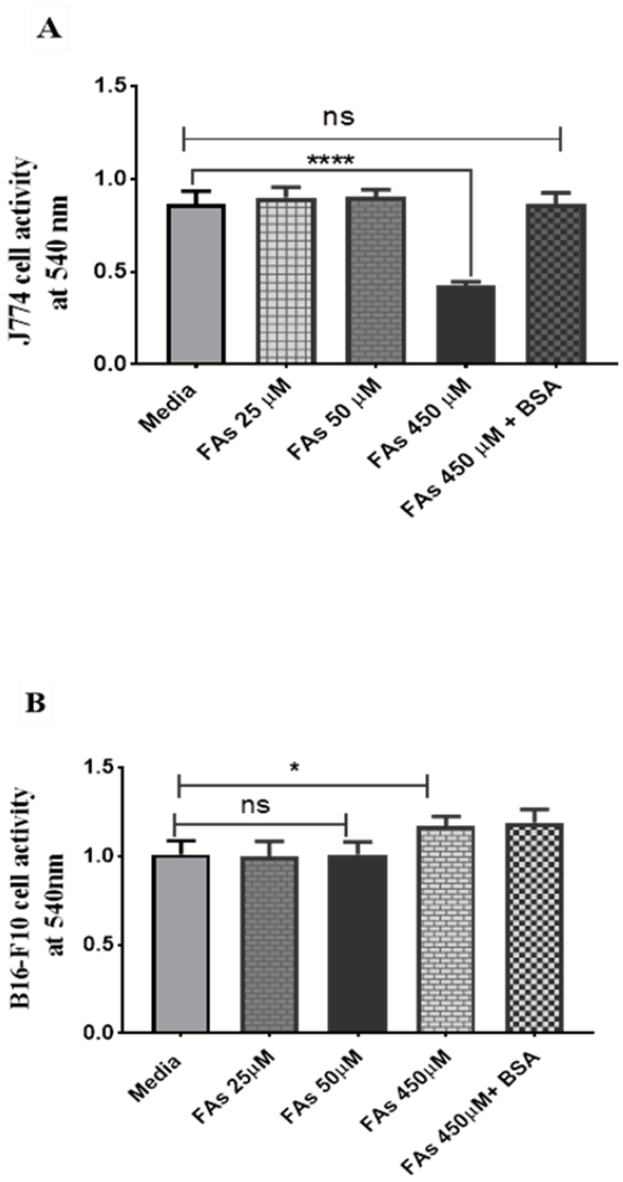


Figure 3-2: Effect of fatty acids supplementation on cell survival of murine macrophages (J774) and melanoma cells (B16-F10).

(**A**) The effect of FAs on cell viability of J774. (**B**) The effect of FAs on cell activity of B16-F10. **C**ells were cultured in DMEM containing different concentrations of FAs (25, 50, 450 and 450 μM with BSA) or of FAs mixture (Oleic and palmitic at a ratio of 2:1) for 24hrs. Cell viability was determined by Crystal violet assay at 450 nm absorbance. Data were presented as mean ±SEM of three independent experiments (n=3) and each samples was measured in triplicate. Statistical analysis was performed by one-way ANOVA/multiple comparison test. ($^{*}p< 0.05$).

3.3.2 Morphological adaptation of murine melanoma cell line (B16-F10) to FAs incubation

This experiment aimed to investigate the effects of FAs on mouse skin melanoma cell line (B16-F10) and to examine whether FAs could induce lipid accumulation of neutral triglycerides in these cells. Light microscopic examination revealed that in comparison to untreated cells, FAs treated cells at higher concentration (450µM) for both periods (24 and 48 hours) had changed to increase their size, cell membrane exhibiting finger-like projections and the inclusion of intracellular vacuoles. These morphological changes were more prominent in cells stimulated with 450 µM for 48 hours (**Figure 3-3C and E)** compared with 24 hours (**Figure 3-3A)**, and contrasted with the clear cytoplasm observed in untreated cells (**Figure 3-3B, D and F**). Lower FAs concentrations (25 µM and 50 µM) did not yield any vacuole inclusion (data not shown).

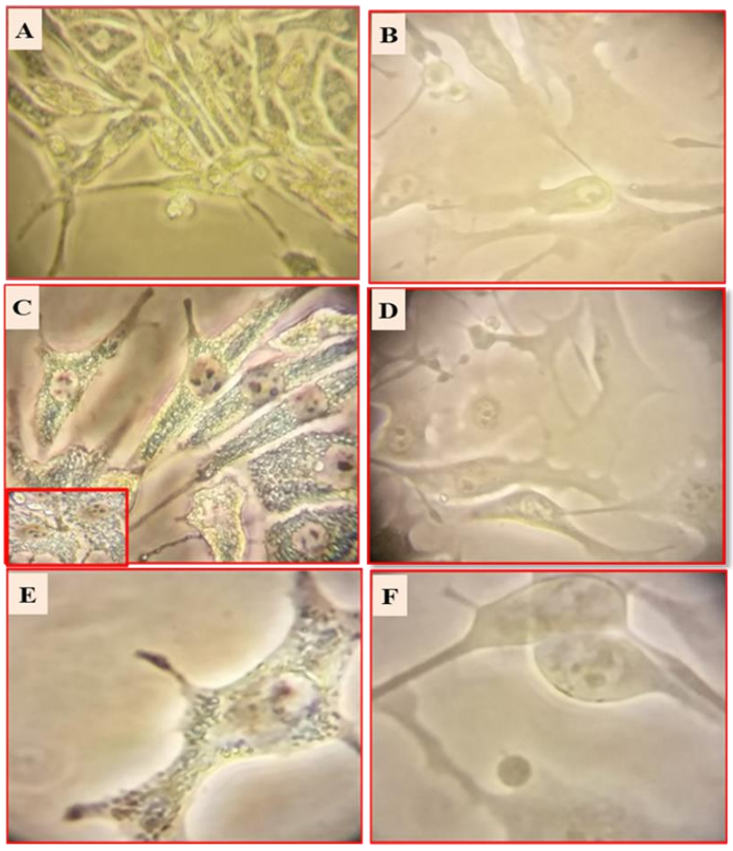


Figure 3-3: Representative pictures showing formation of intracellular vacuoles in murine melanoma cell line (B16-F10).
(**A** and **C**) show a marked increase in cellular size with prominent vacuoles formation on B16-F10 after treatment with 450µM FAs at 24 and 48 hours, respectively. (**B** and **D**) show the absence of these vacuoles in untreated cells under (10x) magnification. (E and F) show presence and absences of these vacuoles under high magnifications (40x).

3.3.3 Fatty acids are associated with morphological changes in murine macrophages (J774)

The effect of FAs (combination of oleic acid and palmitic at (2:1) on cultured murine macrophages was determined at different time points. Treated cells with BSA-Fatty acids mixture for 24 hours had an increase in their cellular size, formation of vacuoles and membrane alteration (**Figure 3-4B**), compared to control cells **(Figure 3-4A).** However, these morphological changes were more prominent in cells stimulated with 450 µm at 48 hours (**Figure 3-4C**). The results also found that lower FAs concentrations (25 µM and 50 µM) had no visible effect on the cells (data not shown).

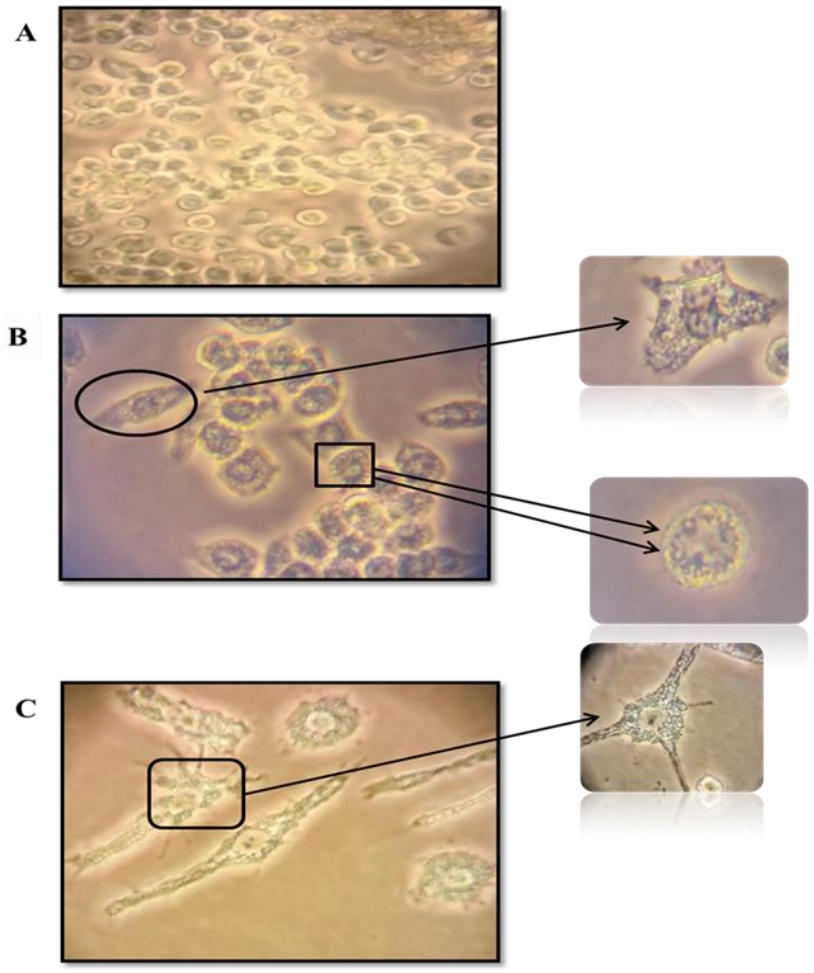


Figure 3-4: Representative photomicrographs demonstrating vacuole accumulation in murine macrophages cell line (J774).
Untreated murine macrophage cells **(A**) compared with increased in cellular size and formation of intracellular vacuoles in J774 after treatment with BSA-fatty acids mixture for 24 hours (B). (**C**) Striking changes in cell membrane and massive vacuoles formation after 48 hours at the same concentration. Magnifications (40X).

3.3.4 Investigation of lipid inclusion in B16-F10 by Oil Red O staining

To determine the nature of the vacuoles inclusion, Oil Red O staining was carried out on B16-F10. The inclusion in J774 has previously been shown to be positive for oil red O when stimulated with FAs (PhD thesis Kheder, 2017). The stain demonstrated lipid accumulation in the mouse skin melanoma cell line at 450µM at 24 hours which was characterized by the appearance of vast oil red O spots (**Figure 3-5A**) compared to their control (**Figure 3-5B**). However, cells under the same concentration at 48 hours showed massive lipid droplets (**Figure 3-5C**), compared to medium treated cells (**Figure 3-5D).**

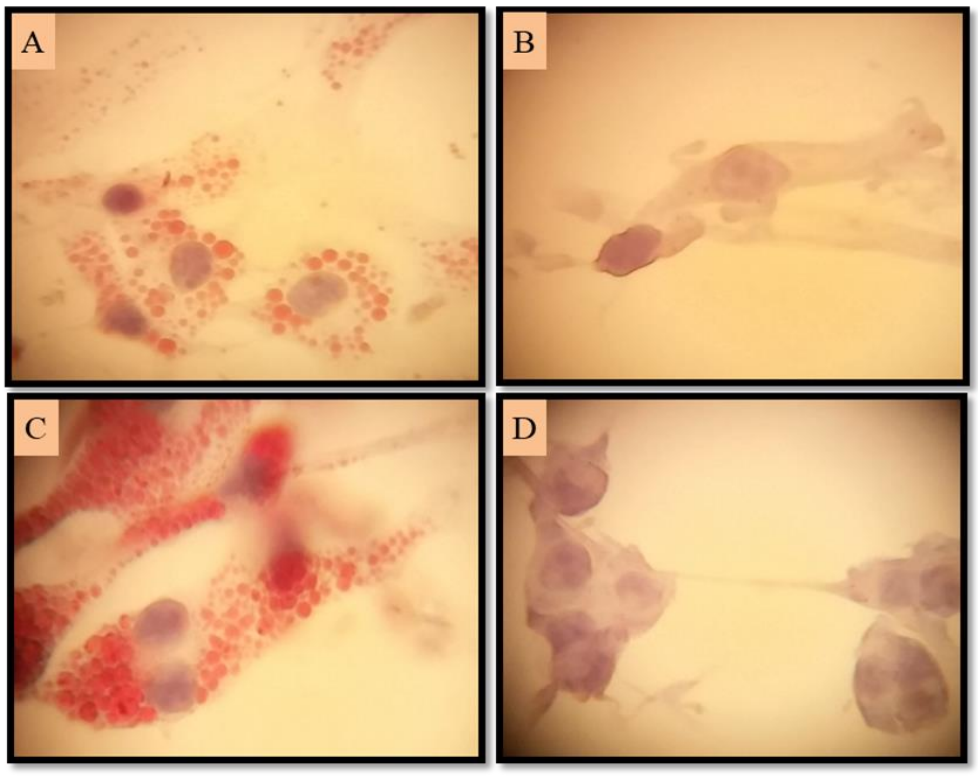


Figure 3-5: staining of natural lipids in murine melanoma cell line (B16-F10) by Oil red O stains.

(**A**) Accumulation of fat drops stained in red colour after treatment with 450µM at 24 hours compared to their control (**B**).While, (**C**) Increased in lipid droplets under the same concentration at 48 hours in comparison with untreated cells with clear cytoplasm (**D**). Magnifications (40x).

3.3.5 Fatty Acids enhance melanoma cells (B16-F10) migration

Motility is a fundamental feature of live cells, cancer cell migration considered as a major cause of death and this is specifically related to metastatic progression of tumour (Hulkower and Herber, 2011). Cancer cell migration and invasion are the hallmarks of malignancy and targeting cancer cell motility is an important therapeutic target (Paul *et al.*, 2017). Migration mechanisms can be induced by various signaling pathways and regulatory networks through cell-cell matrix adhesion and interaction with tumour environment (TEM) (Yilmaz and Christofori, 2010). Study cell migration is very useful and important for cancer immunology. It investigates the ability of cancer cells to sense a particular chemo-attractant and migrate toward it (Calviello *et al.*, 2013). Therefore, a wound healing assay assessed the effect of 450µM of FAs on the migration of murine melanoma cells (B16-F10). The results showed that scratch wounds were almost the same size in each experimental group at 0 hours (**Figure 3-6A and B**). However, cell migration was significantly enhanced (P=0.0002) in cells treated with 450 µM FAs at 24 hours (**Figure 3-6A and B**). Compared with the control, the percentage of healing and wound closure was significantly higher (P =0.0002) in cells treated with FAs (**Figure 3-6C**). These findings clearly indicate that FAs increase the migration and extent of wound closure of B16-F10 cells after 24 hours and, this may be due to enhance secretion of growth factors, chemokines and the activation of specific receptors for fatty acids. Therefore, the effect of FAs on the chemokine profile and on expression of the fatty acid receptor GPR120 was assessed subsequently.

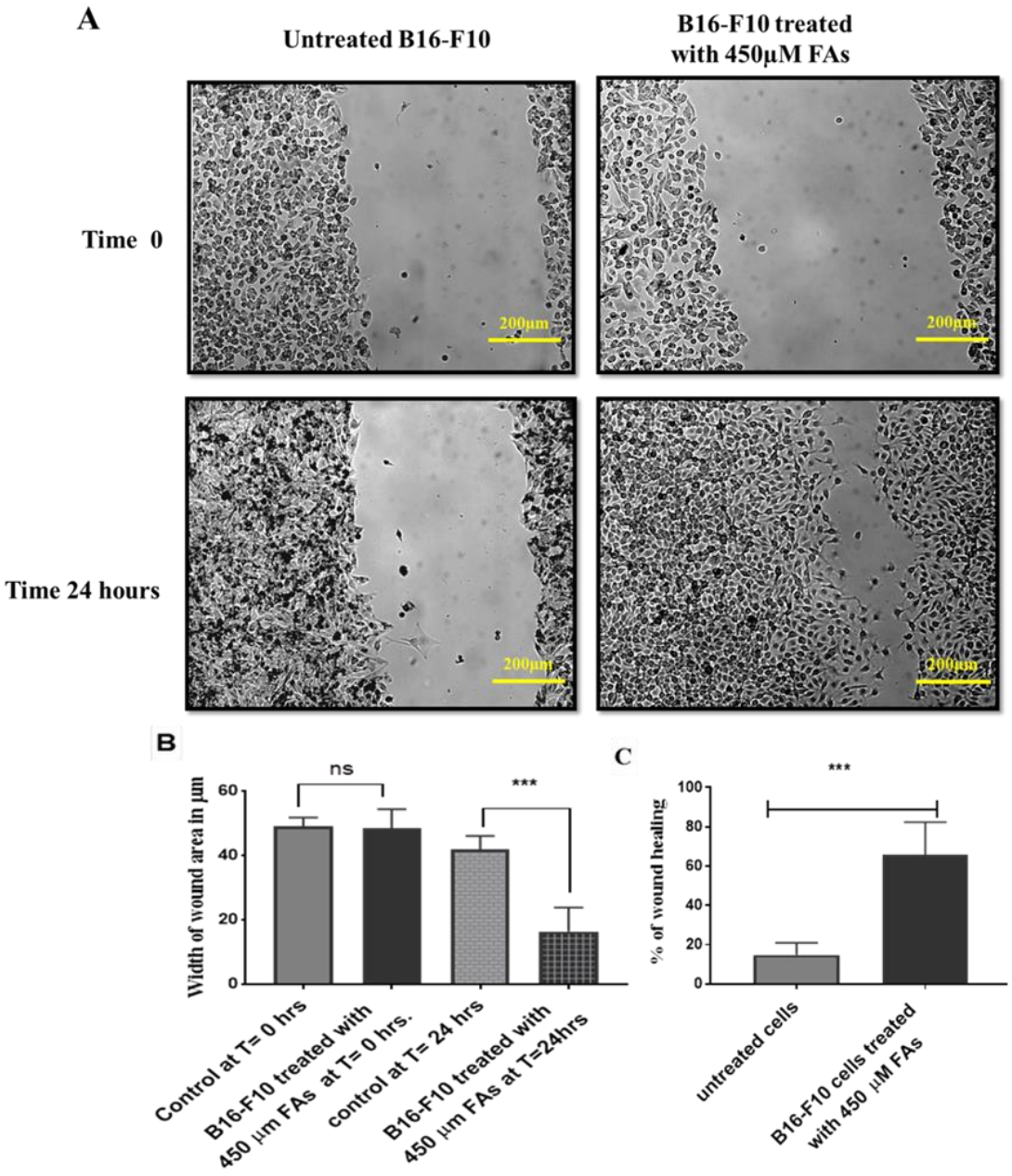


Figure 3-6: Effects of fatty acids on murine melanoma cells migration under different treatments using *in vitro* wound healing assays.

(**A**) Representative images of cell migration were recorded by phase contrast microscopy at 0 and 24 hours' time points after wound scratch (40X) magnification. (**B** and **C**) The level of cell migration into the wound scratch was quantified as the percentage of reduction of initial scratch area and the percentage of wound closer compared to that of control cells. Data are represent as mean ± SEM of three independent experiments and each experiment was done in triplicate n=3 *vs* control. Statistical analysis was performed by one-way ANOVA/multiple comparison test (**B**) unpaired *t* test (C). ($^{*}P< 0.05$).

3.3.6 Fatty acids down-regulate mRNA expression of GPR120 in B16-F10

Previous data showed a significant increase in wound closure after stimulation with fatty acids thus, we proposed that this migration of cells may be due in part to the activation of specific receptors for fatty acid. Therefore, the expression of GPR120 in murine melanoma cell line (B16-F10) was investigated and we determined whether expression was different if these cells were treated with 450 µM FAs at different time points. RT-PCR was done with specific primers for GPR120, which produced a PCR product of 235 base pairs. The results showed that GPR120 mRNA was expressed in all samples (**Figure 3-7A**). Primary liver cells and murine macrophages were used as positive controls for this analysis and GAPDH was utilized as a housekeeping **(Figure 3-7B)**. To determine whether there were quantitative differences across cell lines/treatments, qPCR was employed to more accurately determine expression levels. Quantitative analysis showed that the mRNA expression of GPR120 was significantly down regulated after exposure to 450µM FAs for 24 hours compared to untreated cells (P=0.0141) (**Figure 3-8) and (Figure 3-9)**. However, this expression was highly down-regulated with increased time of exposure at 48 hours compared to both control and cells treated for 24hours (P=0.0069) (**Figure 3-8) and (Figure 3-9)**.

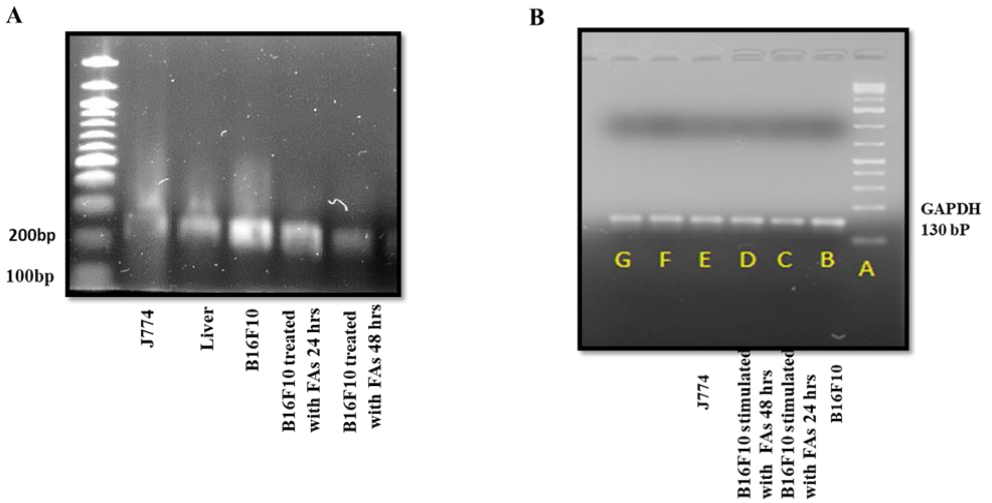


Figure 3-7: Agarose gel electrophoresis of PCR products determining the expression of the GPR120 receptor in murine melanoma cells (B16F10) mRNA.
(**A**) The Photo showed an ethidium bromide-stained gel containing the reaction products following PCR amplification using GPR120 specific primers with expected product size 235 bp. GPR120 was expressed in B16-F10 mRNA under different condition. However, samples treated with 450µM FAs for different period give bright bands compered to untreated cells. (**B**) (GAPDH) was used as a housekeeping gene. The data are representative of two independent experiments n=(2).

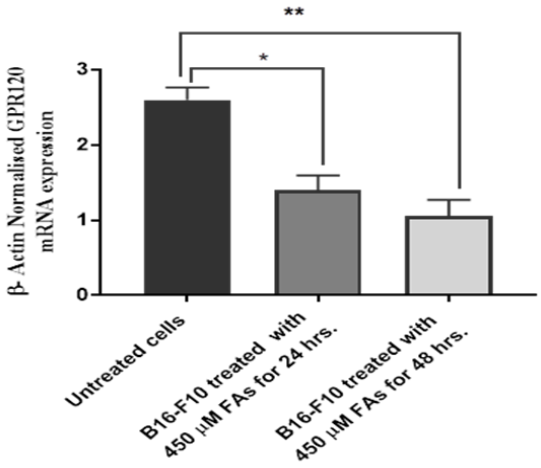


Figure 3-8: Quantitative analysis of GPR 120 mRNA expression in murine melanoma cell line (B16-F10).

Quantitative real time PCR (qPCR) was done to compare the level of gene expression of the GPR120 receptor in B16-F10 at two time points. The mRNA expression corrected for *β*- Actin as reference gene and compared with untreated cells. Data are represented as mean ± SEM of three independent experiments and each experiment was done in triplicate n=3. Statistical analysis was performed by One-way ANOVA/multiple comparisons test. ($^{*}P< 0.05$).

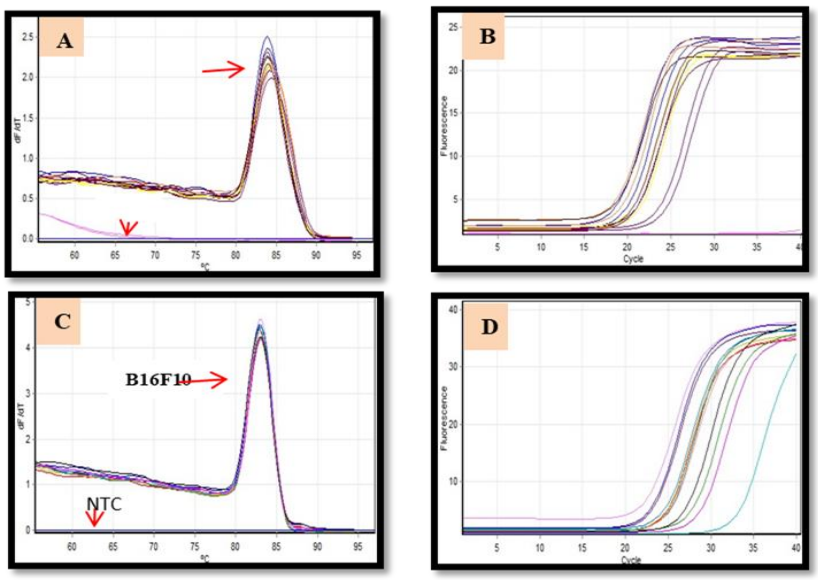


Figure 3-9: SYBR Green-based amplification and melting curve for receptor GPR120 expression in melanoma cancer cells (B16-F10).

(**A** and **B)** the melting curve and amplification curves analysis of expression of *β*- Actin (housekeeping gene). Temperature was plotted against dF/dT [rate of change of fluorescence in the reaction with time (T)]. While, (**C** and **D**) Representative curves for GPR120 receptor. Each assay target is represented by a different colour and NTC indicates non-template control. Liver tissue was used as positive control and duplicate reactions were run for each sample.

3.3.7 Serum from High Fat Diet mice increases cell viability of B16-F10

This experiment aimed to investigate whether circulating factors present in high fat diet fed (HFD) mice could influence melanoma cell proliferation *in vitro*; cells were treated with 2% serum in (free FCS medium) derived from *LDLR*$^{-/-}$ mice fed on HFD or CD mice for 24 hours and then photometric MTS were performed to detect mitochondrial cell activity.There was significant increase in mitochondrial cell viability of cells exposed to serum derived from mice fed on HFD compared to those treated with serum from control diet (low fat diet) (*P*= 0.0158) (**Figure 3-10**).

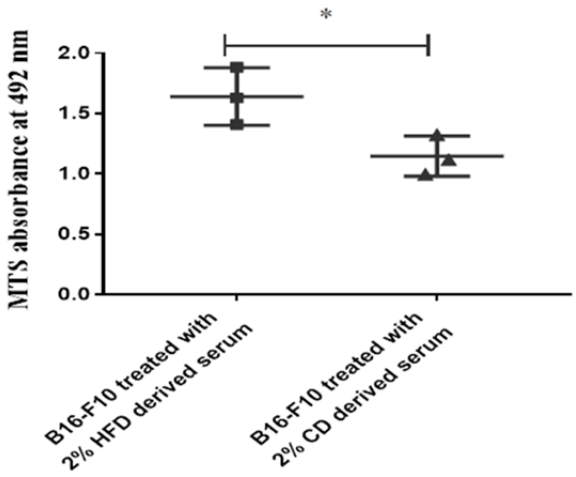
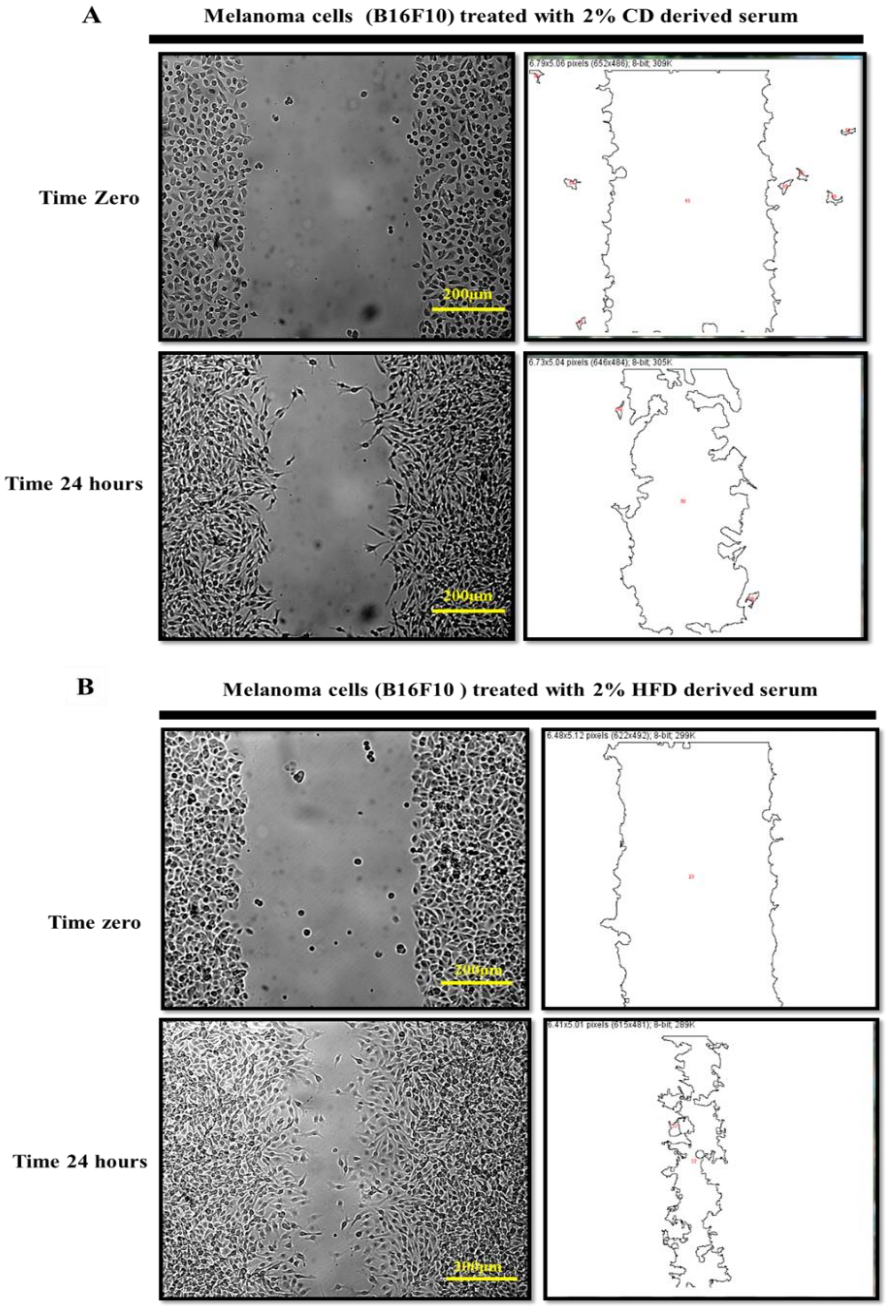


Figure 3-10: Quantification of B16-F10 cells viability after exposure to 2% CD or HFD derived serum.

Mitochondrial activity was used as an indicator of cell viability by MTS and expressed as photometrical densities of the solubilised product formazan. The data are represented as mean ± SEM of three independent experiments and each experiment was done in triplicate (n=3). Data analysis was performed by unpaired t test. ($^{*}P< 0.05$).

3.3.8 Serum from High Fat Diet mice enhances B16-F10 migration using wound healing assay

The effect of 2% serum derived from *LDLR*$^{-/-}$ mice fed on HFD or CD on mouse skin cancer (B16-F10) migration was investigated using wound healing assay. The results showed that at the start of the experiment, the area of scratch wound nearly the same in each test group (**Figure 3-11A and B**). However, after 24 hours the cell migration was significantly (*P*= 0.0079) enhanced in cells treated with HFD derived serum with ~ 60% wound healing compared to their counterpart treated with CD with ~40 % wound healing (**Figure 3-11A, B and C**).



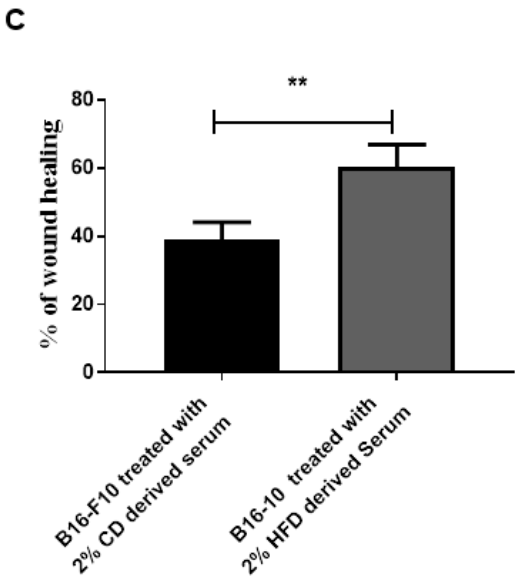


Figure 3-11: Migration of murine melanoma cells (B16-F10) treated with either HFD or CD derived serum.

(**A** and **B**) Representative photos of wound sealing at 0 and 24 hours after wound scratch for cells treated with CD or HFD derived serum respectively. Cell migration was recorded by phase contrast microscopy over a 24 hours' time course after wound scratch. The level of cell migration into the wound scratch was quantified as a percentage of wound healing and compared against control cells at each time point. Data are represent as mean ± SEM of three independent experiments (n=3) and each experiment was done in triplicate *vs* control. Statistical analysis was performed by unpaired *t* test. ($^{*}P< 0.05$, $^{**}P < 0.01$).

3.3.9 The impact of exogenous addition of IL-6 and IL-10 in HFD or CD derived serum on B16-F10 viability

As explained in the introduction, enlarged adipose tissue leads to a dysbalance of pro-inflammatory and anti-inflammatory factors secreted by adipose tissue, and thereby promoting inflammation, impairing insulin sensitivity and dysregulating lipid metabolism (Gutierrez *et al.*, 2009). Therefore, it was hypothesised that pro-inflammatory IL-6 in addition to factors presence in serum from mice fed on HFD would accelerate B16-F10 cell viability. By contrast, adding anti-inflammatory recombinant IL-10 in to HFD serum would have the opposite effect on B16-F10 cell proliferation. Moreover, we tested the effect of adding these two cytokines into the low fat diet serum. There was highly significant reduction in cell viability in cells treated with 2% CD serum containing recombinant IL-10 compared to cells treated with CD serum only (**Figure 3-12A)**. Furthermore, cell viability was significantly induced in the presence of recombinant IL-6 in serum derived from mice fed on a CD (**Figure 3-12A)**.

Interestingly, B16-F10 cells treated with HFD containing recombinant IL-6 showed a significantly greater increase in mitochondrial cell activity compared to those treated with 2% HFD serum (**Figure 3-12B**). Conversely, there was a reduction in cell viability in cells treated with recombinant IL-10 containing HFD serum (**Figure 3-12B**).

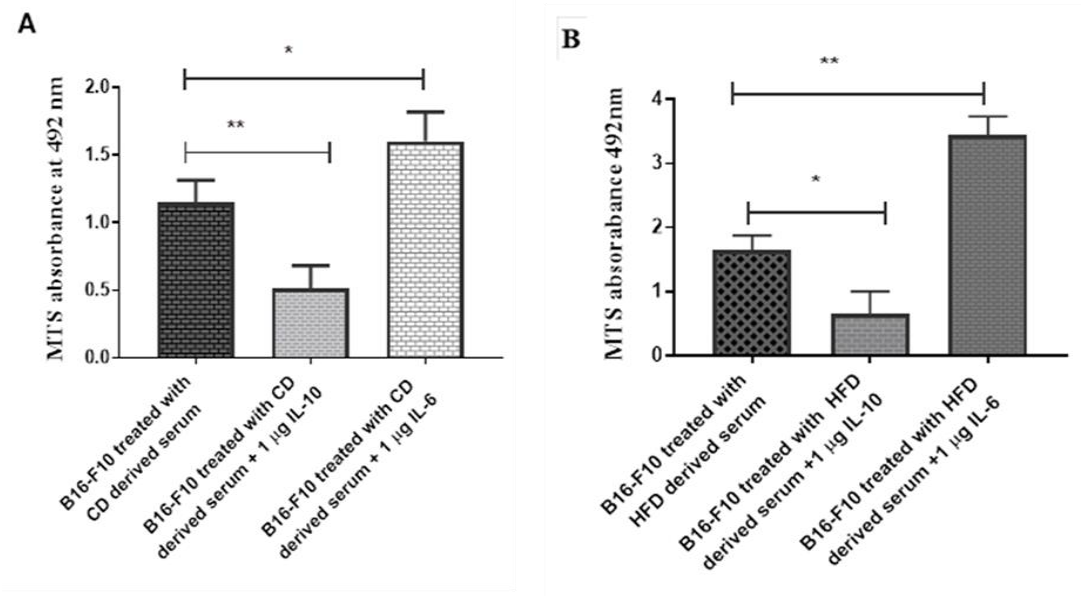


Figure 3-12: Measurements of mitochondrial cell activity of B16-F10 treated with exogenous addition of IL-10 or IL-6 in CD or HFD derived serum.

Colorimetric MTS was performed to measure the mitochondrial cell activity of B16-F10. (**A**) The effect of IL-10 and IL-6 addition in control diet derived serum. (**B**) The effect of exogenous addition of recombinant IL-10 and IL-6 in HFD derived serum on (B16-F10) viability. The data are represented as means of triplicates ± SEM (n=3), and the data analysis was performed by one-way ANOVA/multiple comparisons test. ($^{*}P< 0.05$).

3.3.10 Fatty acids down-regulate expression of surface Toll-like receptor- 4 in (B16-F10)

Inflammation has an undisputed role in cancer development and progression. TLR-4 is expressed not only on immune cells, but also on tumour cells such as human lung cancer cell lines A549 and H460 (Zhan *et al.*, 2014), human cervical squamous epithelial cells (Yu *et al.*, 2010), and colon cancer cells (Wang *et al.*, 2010). Previous studies have revealed that adipose tissue expansion during obesity dysregulated the production of pro-inflammatory or inflammatory cytokines, obesity could be considered as a chronic inflammatory disease triggered by fatty acids which is act as DAMP molecules (Jounai *et al.*, 2012). Clinical trials clearly demonstrated that a key mediator that links high fat (HF) diet-mediated inflammatory responses to the pathogenesis of obesity and insulin resistance, is the activation of toll-like receptor 4 (TLR-4) by fatty acids (Okla *et al.*, 2015). Therefore, we hypothesised that fatty acids have the ability to modulate the expression of TLR-4 on murine melanoma cells (B16-F10). Surface expression of TLR-4 was significantly down-regulated after treatment with 450µM FAs for 48 hours when compared to untreated control cells (**Figure 3-13**). Murine macrophages (J774) were used as positive control. Cells were stained with labelled anti-TLR-4 PE and analysed by flow cytometry (**Figure 3-14**).

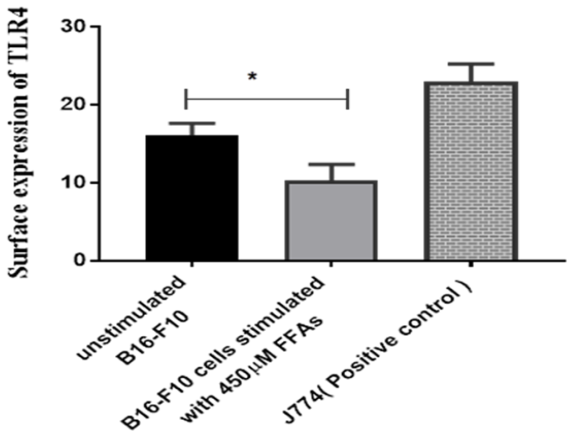


Figure 3-13: Expression of TLR-4 on murine melanoma cell line (B16-F10) by Flow cytometry analysis

Cell surface expression of (TLR-4) was analysed by FC after staining with a goat anti-mouse TLR-4 PE. Untreated B16-F10 cells were used as a control while murine macrophages were used as positive control. Data are represented as mean ± SEM of three independent experiments (n=3) *vs* control and statistical analysis was performed by One-way ANOVA/multiple comparisons test. ($^{*}p < 0.05$).

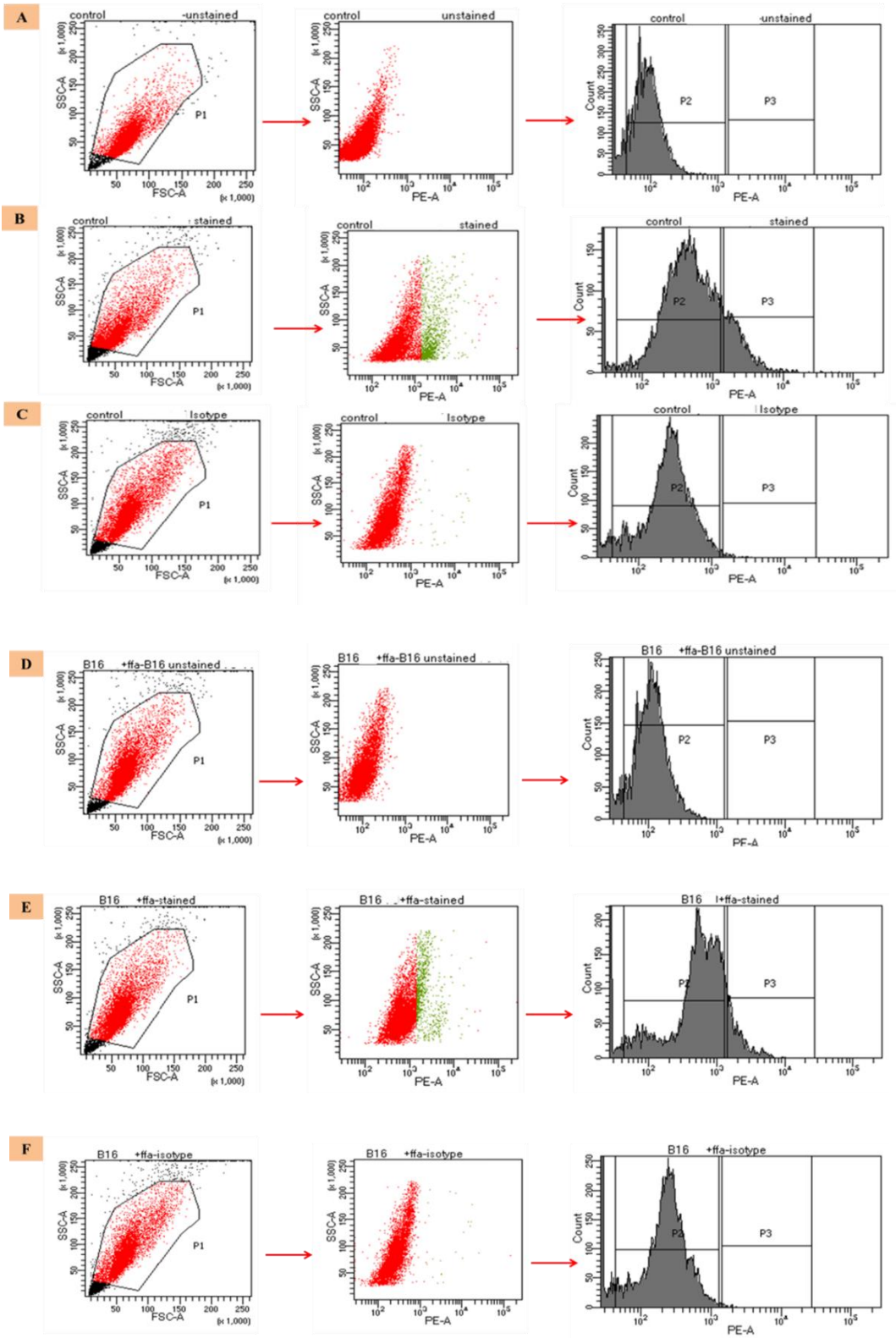


Figure 3-14: Representative flow cytometry histograms showing surface expression of TLR-4 on B16-F10 melanoma cells.

(**A**, **B**, and **C**) Fluorescence histogram for unstained, stained and isotype respectively for untreated cell (control). While, (**D, E** and **F**) Expression of TLR-4 in B16-F10 treated with FAs. (**G, H** and **I**) fluorescence histogram for murine macrophage cells (J774) (positive control).

3.3.11 Fatty acids up-regulate chemokines secretion in B16-F10

Chemokine gradients within the tumour microenvironment and/or in the blood circulation and lymphatic system play an important role in cancer cell migration (Psaila and Lyden, 2009). Our data showed a significant increase in cell migration at 24 hours and so it was important to investigate the effect of FAs on chemokines expression in B16-F10 cells. A commercially available mouse chemokine array kit was used to determine the relative changes in a panel of mouse chemokines (n=25) in B16-F10 supernatants after exposure to 450µM FAs for 48 hours to identify differences in chemokines secretion in response to FAs treatment. The time point and concentration were chosen based on previous experiments which showed high accumulation of FAs under these conditions Proteomic analysis showed that 11 out of the 25 candidate chemokines has altered expression (**Figure 3-15B**).

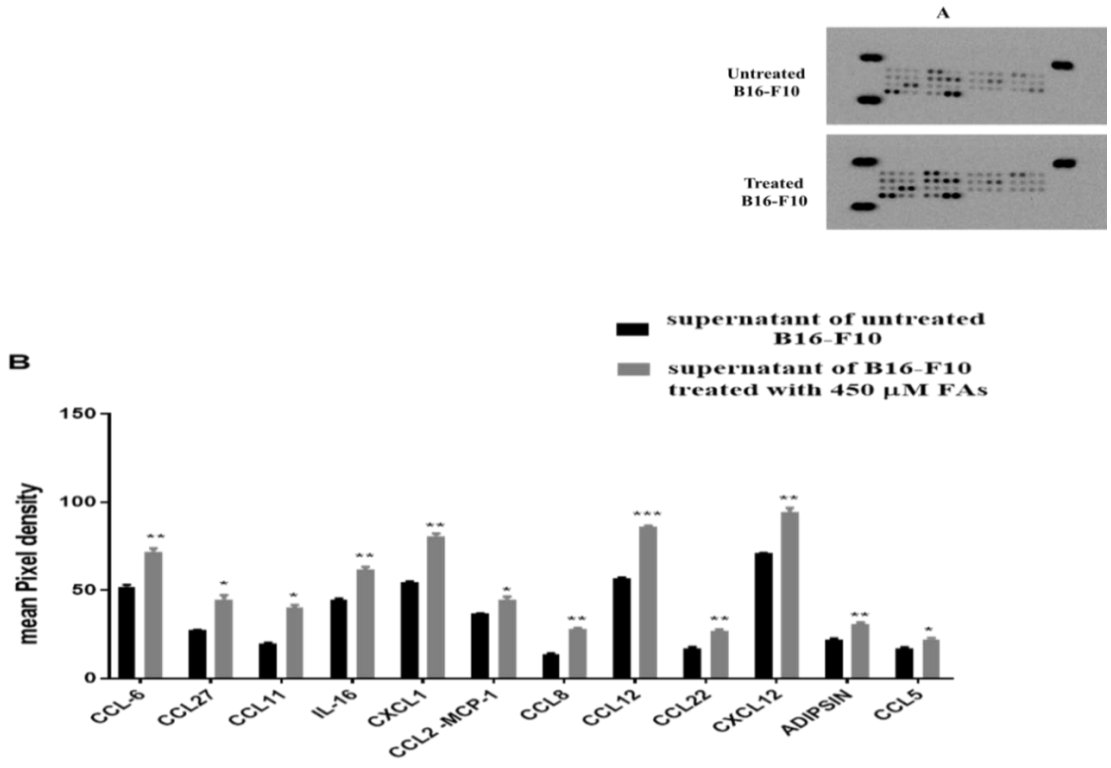


Figure 3-15: Proteomic analysis of murine melanoma cell (B16-F10) treated with FAs.
(**A**) Array membranes from five minutes exposure to ESL and light signals were detected by Bio-Rid machine. The high-intensity spots in the three corners of each array represent the positive control. (**B**) Antibody -based protein arrays were used to assess the presence of 25 chemokines in B16-F10 supernatant after treatment with FAs for 48 hours. However, CXCL13, CCL28, CHEMERIN, CX3CL1, CXCL11, CXCL10, CCL9/10, C5/C5a, CXCL16, SRPSOX, CCL11, CCL3/CCL4, CXCL2, were not detected in treated cells compared to untreated cells. The data are presented as means ± SEM. Statistical analysis was performed by Two-way ANOVA Sidak's multiple comparisons test. ($^{*}P< 0.05$).

3.3.12 Serum from High Fat Diet mice induces expression of leptin receptors (ObRs) in B16-F10

The expression of leptin receptor (ORs) was investigated in murine melanoma cell line (B16-F10) under different conditions. B16-F10 cell lysates were prepared from cell stimulated with either 2% serum derived from mice fed on HFD or control CD for 48hrs and then subjected in 10 % SDS PAGE. Western blot analysis showed expression of all leptin isoform (ObRs) in all experimental lysates. However, this expression was less in cells treated with 2% HFD serum compared to CD serum. (**Figure 3-16**).

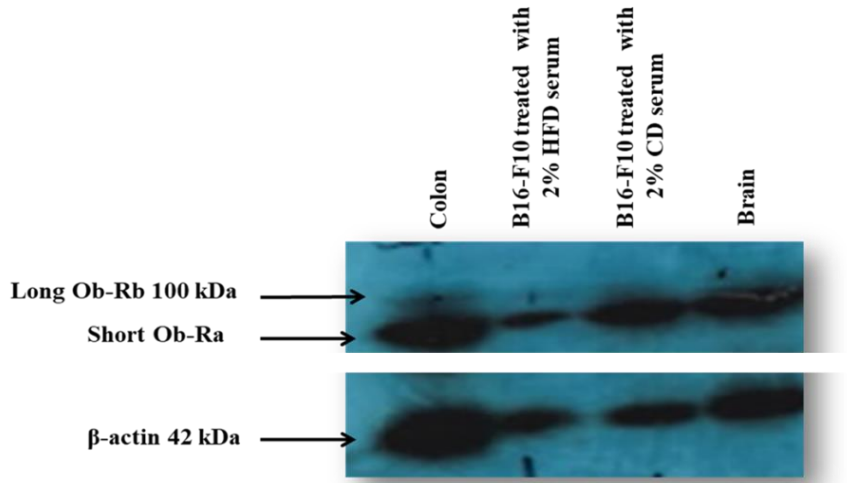


Figure 3-16: Immunoblot of Leptin receptor isoforms (Ob-Rs) in murine melanoma cell line (B16-F10).
Lysate from cells treated with either 2% serum derived from mice fed on high fat diet or control diet were analysed by western blotting with indicated antibodies. 20 µg of each lysate were subjected in 10 % SDS PAGE under reducing conditions, β-actin was used as loading control and Brain and Colon tissue lysates were used as positive controls. The data are representative of two independent experiments (n=2).

3.3.13 Fatty acids induce pro-inflammatory responses in murine macrophages (J774)

To investigate the effect of FAs as pro inflammatory activators of macrophage activity the effects of 450 µM BSA-fatty acid mixture on the activity of J774 macrophages was assessed. The 450 µM of FAs corresponds to the concentration of free fatty acids in blood in obese individuals (normal level: 160- 480 mmol/L) (Shiomi and Watanabe, 2013). Macrophages treated with 450 µM BSA-fatty acid mixture significantly induced cells to secrete the pro- inflammatory cytokine TNF-α compared to control cells **(Figure 3-17**). Moreover, the results also indicate that the cells co-cultured with cell condition medium of B16-F10 significantly enhanced macrophages to secrete TNF-α and this may be occurred via TLR-4.

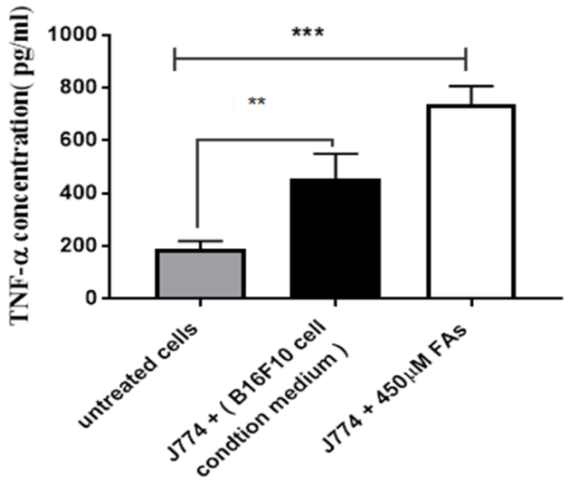


Figure 3-17: Detection of TNF-α in the supernatant of murine macrophage cell line (J774). The ELISA method was used to detect TNF-α in J774 cell culture supernatant after treatment with 450 µM of (combination of oleic acid and palmitic at 2:1) BSA- mixture for 48 hours and pre-cluttered with cell condition medium of B16-F10 for the same period. Data are mean ± SEM of three independent experiments (n=3) *vs* control and statistical analysis was performed by One-way ANOVA/multiple comparisons test. ($^{*}P< 0.05$).

3.3.14 Cell conditioned medium of B16-F10 modulates chemokine CCL2 (MCP-1) production in macrophages

To investigate whether tumour cells induce macrophages to produce CCL2, J774 macrophages were incubated with conditioned medium of B16-F10 melanoma cells. The results showed a significant increase in CCL2 secretion from J774 cells after incubation with cell conditioned medium of B16-F10 compared to J774 cells alone. Murine B16-F10 tumour cells produced little CCL2 in comparison to macrophages *(P*= 0.0075) (**Figure 3-18**). These results clearly indicate that murine macrophages are by far the main producers of CCL2, compared to tumour cells.

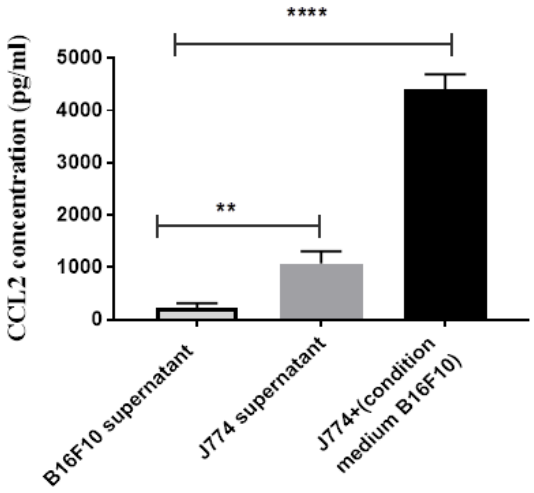


Figure 3-18: Effect of B16-F10 conditioned medium on CCL2 secretion by J774 cells.
The ELISA method was used to detect CCL2 protein in J774 cell culture supernatants after incubation with cell conditioned medium from B16-F10 treated for 48hrs. Data are mean ± SEM of three independent experiments (n=3) and each sample was tested in triplicate. Statistical analysis was performed by one-way ANOVA/multiple comparisons test. (*$p < 0.05$).

3.3.15 Fatty acids and conditioned medium of cancer B16 up-regulate CCL2 derived macrophages secretion

The CCL2 chemokine (MCP-1) is a major chemoattractant of macrophages to tissues and its role in melanoma growth is widely investigated (Li *et al.*, 2013). We proposed that tumour resident macrophages would produce CCL2 to encourage further recruitment of macrophages and other CCL2 receptor binding immune cells. Therefore, the experiment aimed to analyse the induction of macrophage derived CCL2 by FAs and tumour cells. There was significant increase in CCL2 production from macrophages incubated with FAs and cell conditioned medium of B16-F10 compared to other conditions (P= 0.0377) (**Figure 3-19**). However, no significant difference was seen on Mϕ-MCP-1 secretion between macrophages treated with FAs mixture and those treated with cell conditioned medium of B16-F10 only.

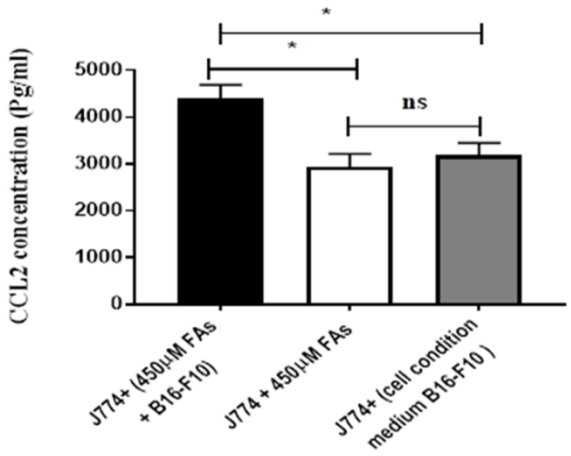


Figure 3-19: The effect of combined actions of FAs bound-BSA and tumour cell supernatants on macrophage derived CCL2.
Production of protein CCL2 in the supernatant of macrophages cells (J774) under different conditions was investigated by ELISA method. Data are represented as the mean ± SEM of three independent experiments (n=3) and each sample was tested in triplicate. Statistical analysis was performed by One-way ANOVA/multiple comparisons test. (*$p < 0.05$).

3.3.16 Fatty acids and tumour cells supernatants enhance murine macrophage migration

Previous data showed that murine macrophages treated with FAs and cell conditioned medium of melanoma cancer cells (as *in vitro* model of cancer in cause of obesity) significantly enhanced to secrete TNF-α and Chemokine MCP-1 (CCL2). Therefore, it was important to investigate the effects of fatty acids and tumour conditioned media on macrophage (J774) cell migration. The hypothesis was that cancer cell secreted factors in the presence of fatty acids would enhance macrophage migration. The results showed that macrophage migration after 24 hours was significantly higher in cells treated with FAs and cell conditioned medium of melanoma cells (B16-F10) compared to controls and other conditioned (p=<0.0001) **(Figure 3-20)**. Furthermore, wound healing assay also found that FAs significantly enhanced cell migration after 24hours compared to control and cell conditioned medium of B16-F10 (p=0.0025) **(Figure 3-20).**

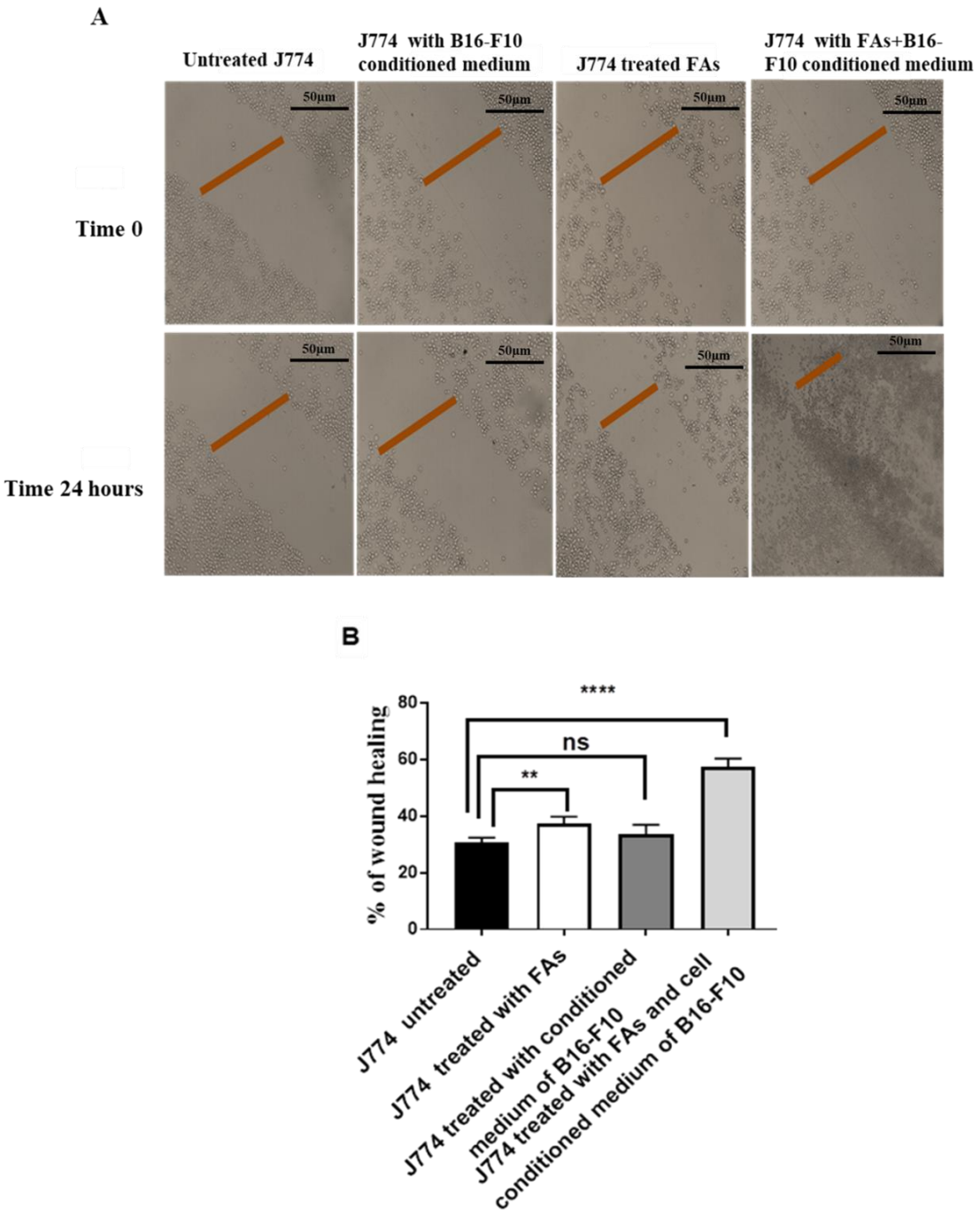


Figure 3-20: Representative images showing *in vitro* migration of murine macrophages using wound healing assay.

Images of cell migration were taken immediately after wound scratch at 0 times and after 24 hours. The data are represented as the mean ± SEM of three independent experiments (n=3). Statistical analysis was performed by one-way ANOVA/multiple comparisons test. (*$p < 0.05$).

3.3.17 Fatty acids and conditioned medium of cancer cells differentially regulate macrophage activities

Tumour microenvironment signals are a key regulator of macrophage functions, which drive the acquisition of polarised programmes. Two distinct macrophage activation states have been recognized, M1 and M2. Functional skewing of monocyte/macrophage polarization occurs in pathological states such as chronic inflammation, tissue repair, and cancer and is considered key determinant of disease development and/or regression (Sica *et al.*, 2015).

Our ELISA data previously showed that combined actions of FAs mixture and cell conditioned medium of B16-F10 significantly induced chemokine CCL2 secretion from macrophages. Therefore, recent experiment suggests that fatty acid may stimulate pro-inflammatory pathway activity in murine macrophages and thereby polarise them to M1 or M2 phenotype macrophages. The results showed that cell conditioned medium from B16-F10 supplemented with 450µM FAs significantly enhanced murine macrophages (J774) expression of iNOS compared to both controls and cells treated with conditioned medium from tumour cells (**Figure 3-21A**). These results indicate that FAs may drive macrophages towards an M1 phenotype by increasing iNOS mRNA expression. In contrast, cells stimulated with conditioned medium of B16-F10 supplemented with 450µM FAs significantly expressed mRNA Arginase-1 compared to both untreated cells and cells treated with FAs (**Figure 3-21B)**. However, expression of Arginase -1 mRNA was significantly higher in cells treated with cell-conditioned medium of B16-F10 (**Figure 3-21B**).

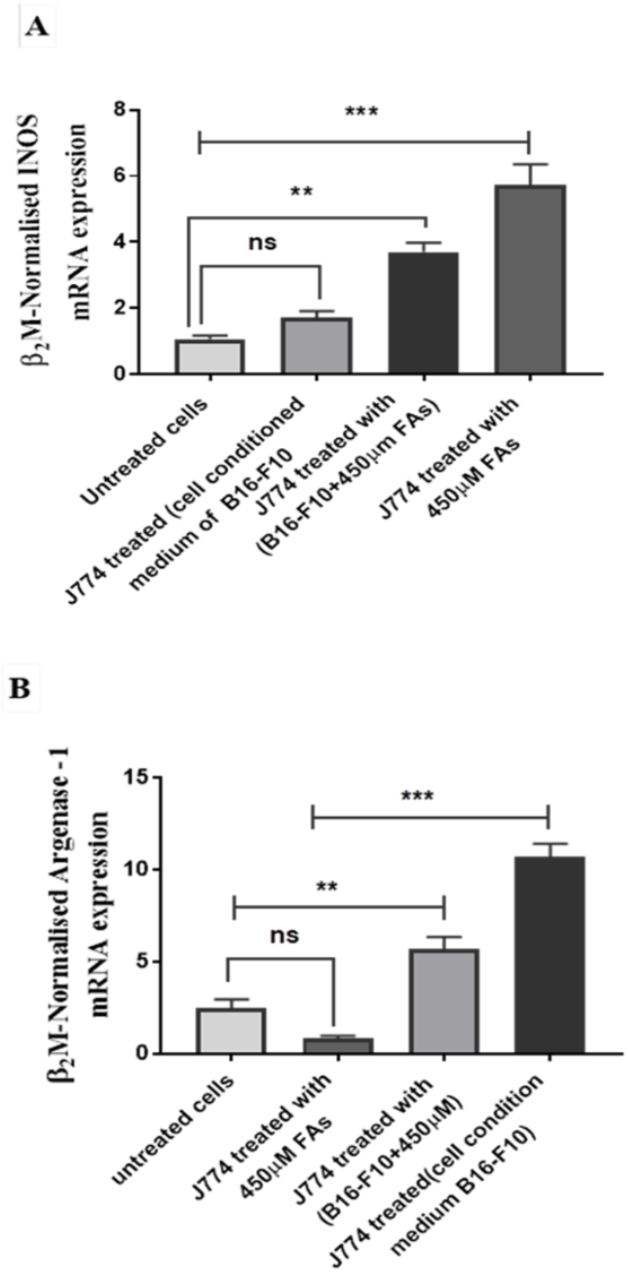


Figure 3-21: Effect of FAs and B16-F10 cell conditioned medium on macrophage activates. mRNA was prepared for quantitative real time PCR (RT—qPCR) to compare the level of gene expression of (iNOS) as a pro-inflammatory M1 marker and (Arginase1-) as anti-inflammatory M2 marker in macrophages under different conditions. ΔΔCT value was used, the mRNA expression corrected for β2-M and compared with untreated cells. The data are represented as the mean ± SEM of three independent experiments (n=3) and each sample was tested in duplicate. Statistical analysis was performed by one-way ANOVA/multiple comparisons test. ($^{*}p < 0.05$).

3.4 Discussion

Alterations in fatty acids (FAs) metabolism in cancerous cells have received less consideration but are increasingly being recognized. Fatty acids can be either saturated or unsaturated based on carbon numbers in hydrocarbon chain and, they are required for energy storage, membrane proliferation, and the generation of signalling molecules. (Currie *et al.*, 2013). Increased plasma non-esterified fatty acids (NEFAs) in obese individuals are considered as an important indicator for complication related –metabolic syndrome (Wang *et al.*, 2009).

The overall purpose of this study was to investigate the effect of FAs on two important players within the tumour microenvironment (macrophages and melanoma cancer cells). To our knowledge the direct *in vitro* effect of fatty acids on the interaction of macrophages and melanoma cancer cells has not been studied before. A combination of two types of fatty acids (oleic acid and palmitic 2:1) was chosen for this work. This is because they are the most abundant dietary and plasma FAs (31% and 27% respectively of total plasma NEFA) and are at a ratio of 2:1 oleic to palmitic acid to minimize the toxic effect of palmitic acid (Palomer *et al.*, 2018). Furthermore, the high concentration of fatty acids (450µM) was used in this study to be comparable with the levels of NEFA in the blood of obese mice (normal range: 160- 480 mmol/L) (Yao *et al.*, 2011; Shiomi and Watanabe, 2013).

The cytotoxic effect of the FAs on cultured cells was determined by crystal violet assay. The results showed that higher concentration of fatty acids significantly enhanced melanoma cell viability at 24 hours. However, this concentration was found to be toxic for murine macrophages at the same period, thus, bovine serum albumin (BSA) was added to reduce this effect (Raynor *et al.*, 2015). This observation agrees with previous work of Martins de Lima *et al.* (2006) who demonstrated that macrophages treated with high concentrations of fatty acids (300 µM Oleic and 200 µM palmitic acid) for 24 hours caused cell death by inducing apoptosis and necrosis. Furthermore, previous study showed that co-cultured B16-F10 for 6 hours with palmitic acid at (3–50 µM) stimulates cell proliferation (Kwan *et al.*, 2014). However, earlier study by Andrade *et al.* (2005), found that 200µM of palmitic acid induced loss of membrane integrity in tumour cell (B16-F10) after 24 hours, while, oleic acids at the same concentration had no effect on B16-F10 cell viability.

These findings are partially agreed with our results. The ability of our B16-F10 to challenge the higher levels of palmitic (450 µM) may be attributed in part to the oleic action, which modulates the cytotoxicity effect of palmitic, as all previous study investigate the single effect of palmitic acid on tumour cell proliferation. The effect of 450µM FAs on cellular morphology of macrophages and B16-F10 at different time points (24 and 48 hours) was also investigated. Light microscopy showed that FAs induced an increase in cellular size and the formation of intracellular vacuoles for both cultured cells at 24 hours and these effects were greatly increased with increased in time of exposure. Oil red O staining showed that FAs induced massive accumulation in cytoplasmic lipid droplet in B16-F10 at 48 compared to both 24 hour-treated cells and control cells. In agreement with Kwan and collaborators (2014) found that mouse skin melanoma cells B16-F10 co-cultured with adipocytes for 24 hours had an obvious increase in their cytoplasmic lipid droplet accumulation compared to the control cells as demonstrated by Oil red O staining. Moreover, previous laboratory-based work (PhD thesis Kheder, 2017) found that FAs induced lipid accumulation in macrophages (J774) treated cells.

Metastasis is the main cause of mortality in cancer patients, and includes a multi-step process involving cell adhesion, invasion, and migration (Choi *et al.*, 2017), and inhibition of cancer cells metastasis is a basic strategy in cancer therapy (Gao *et al.*, 2018). The effect of FAs on migration ability of B16-F10 was investigated using wound healing assay. *In vitro* wound healing assay found that FAs significantly enhanced cell migration at 24 hours compared to untreated cells. Additionally, the study utilizes serum derived from *LDLR*$^{-/-}$ mice fed on either high fat or control diet to study the effect of circulating factors presence in sera of these mice on B16-F10 proliferation. The results showed that HFD derived serum significantly influenced melanoma cell proliferation *in vitro* compared to cells treated with CD derived serum. In agreement with our findings, previous work of Chen *et al.* (2016), who demonstrated that proliferation of melanoma cells (B16-F10), was enhanced significantly after treatment with HFD derived serum compared to CD serum treated cells. Furthermore, the results also showed that HFD is able to enhance B16-F10 migration with ~60% healing compared to cells treated with CD with ~ 40% healing. These findings agreed with Jung *et al*. (2015) who found that co- culturing B16-F10 with adipocytes enhanced tumour cell migration. Additional support comes from Chen *et al*. (2016) who found that the migration rate of B16-F10

was higher after exposure to serum derived from mice fed on a high fat diet than control diet serum. Since cell migration, results from the integration and temporal coordination of many different processes, the expression of fatty acid receptor and the secretion of chemokines were investigated. The expression of GPR120 has previously been confirmed in different mouse cell lines (Zhang and Leung, 2014). However, this is the first study that has identified the expression of this receptor in the melanoma tumour cell line (B16-F10). Endpoint PCR revealed that GPR120 was expressed in B16-F10. The biological activity of FAs exhibit through the activation of GPR120 in cells, tissues and animals (Zhang and Leung, 2014; Ichimura *et al.*, 2014). Therefore, we decided to identify whether this expression was affected if the cells were treated with 450 µM FAs over 24 and 48 hours. The results revealed a significant decrease in GPR120 mRNA expression after treatment with FAs for 24 hours compared to untreated cells, however, the decline in the expression of GPR120 mRNA after 48 hours was significantly higher compared to both control, and 24 hours treated cells. In a study conducted by Wu *et al*. (2013), found that *in vitro* activation of GPR120 promoted colorectal carcinoma cell migration which supports our previous observation. Additionally, prior studies found that the expression of GPR120 in colon cancer cells stimulated cell motility and angiogenesis (Fukushima *et al.*, 2016). Furthermore, unsaturated FAs have been shown to enhance proliferation, cell survival, and inhibit apoptosis in two GPR120-expressing human breast cancer cell lines (Zhang and Leung, 2014).

Animal data and epidemiological studies showed an association between increased secretion of cytokines (i.e., adipokines) from adipose tissue and the promotion of inflammation, cell proliferation, and angiogenesis. Leptin, an adipokine which circulates in the blood in proportion to fat mass has been suggested to link obesity with tumour growth and progression (Brandon *et al.*, 2009). However, the direct effect of Leptin in tumour growth is unclear. Leptin are known to bind to multiple isoforms of the Leptin receptors (ObRs) (Mendonsa *et al.*, 2015). Western blot showed expression of all Leptin (ObRs) in the murine melanoma cell line B16-F10. However, this expression was reduced in cells treated with FAs compared to untreated cells. The same observation has been made in work, which showed expression of Leptin receptor (ObR) in B16-F10 cell lines (Brandon *et al.*, 2009).

The effect of pro and anti- inflammatory cytokines on mitochondrial cell viability of murine skin cancer was investigated. MTS assay found that cells treated with IL-6 addition in 2% serum derived from HFD mice significantly enhanced B16-F10 mitochondrial viability compered to both untreated cells and cells treated with IL-6 addition in 2% serum derived from CD mice. However, contrast effect was demonstrated when IL-10 added into 2% serum derived from HFD indicated by significant reduction in mitochondrial activity of B16-F10. Previous publication showed association between elevated plasma levels of IL-6 and aggressive tumours (Deng *et al.*, 2016). *In vitro* study of Ji *et al.* (2011), demonstrated that IL-6 can exert a direct lipolytic effect and induce mitochondrial dysfunction in mouse adipocytes (3T3-L1). Furthermore, in mouse model of cachexia, IL-6 can have a direct effect on mitochondria biogenesis during the initiation of cachexia and inhibition of IL-6 signalling can attenuate the progression of cachexia cancer (White *et al.*, 2012). On the other hand, the direct effect of IL-10 on mitochondrial cell activity has been demonstrated in a study conducted by Ip *et al.* (2017)), which found that absence of IL-10 signalling induced damage and abnormal function in mitochondrial macrophages in a mouse model of inflammatory bowel disease.

The effect of high concentrations of FAs on chemokine secretion in the supernatant of B16-F10 cells was tested with a proteome profile array. Published study reported that tumour cells secreted and responded to chemokines, which facilitate their growth and metastasis to distant sites (Fukushima *et al.*, 2016). Our results showed that FAs significantly up regulate the secretion of chemokine (CCL2 (MCP1), CCL27, CXCL12, CXCL1, CCL6, CCL11, CCL8, CCL12, CCL22, and Adapsin). This is consistent with findings of Sarvaiya *et al.* (2013), also reported that the CXCR4/CXCL12 axis and CCR2/CCL2 play an important role in angiogenesis and metastasis. In ovarian cancer cells, Yang and co-worker (2008), found that the chemokine CXCL12 activates the chemokine receptor CXCR4 on endothelial cells, which promotes endothelial cell migration and proliferation. Our findings also agreed with works that demonstrate the role of CCL27/CCR10 pathway in tumour growth of murine melanoma B16-F10 cells through the activation of PI3K/Akt pathway and by evading the host anti-tumour response Leptin isoforms (Murakami *et al.*, 2003). Furthermore, CCL8 was found to increase the migration of a human melanoma cell line when added as chemoattractant (Barbai *et al.*, 2015). Another supportive finding is the work of Templin *et al.* (2017),

who found secretion of IL-16 in culture supernatants of human myeloma cell lines and its secretion correlates with the proliferative activity of these cells. Moreover, it has been found that Tumour cells have the ability to produce growth-promoting chemokines and to express chemokine receptors. For example, melanoma tumours secrete several chemokines, including CXCL1, CCL2, CCL5 and CCL6 which are involved in tumour growth and development (Chow and Luster, 2014; Cao *et al.*, 2017). Human recombinant CCL11 enhances cell migration/invasion of human ovarian cancer cell lines over 48 hours (Levina *et al.*, 2009). Interestingly, we show the differential expression of complement factor D (Adipsin) in the supernatant of B16F10 and this expression was significantly higher in cells treated with FAs. The Adipsin /C3a pathway plays multiple roles, such as immune regulation, cell signalling and cell migration (Carmona-Fontaine *et al.*, 2011). The effect of FAs on expression of TLR-4 was investigated in B16-F10 using flow cytometry. FC analysis revealed significant reduction in the expression of surface TLR-4 in cells treated with FAs compared to untreated cells. TLR-4 activation on cancer cells upon inflammation may enhance tumour progression and it is one of main causes of inflammation -induced cancer (Dana *et al.*, 2017). It has been suggested that TLR-4 signalling has a pro-carcinogenic role and TLR-4 can activate protein kinase (MAPK) and NF-*κB* pathways which promote the induction of pro-inflammatory cytokine signalling pathways in a tumour promoting microenvironment increasing proliferation and inhibiting apoptosis of tumour cells (Korneev *et al.*, 2017). Additionally,TLR-4 deficient mice developed reduced inflammatory cytokine secretion in response to diet -induced obesity (Shi *et al.*, 2006). It has been suggested that excessive release of Fatty acids in chronic inflammatory disease such as obesity can act as DAMP molecules to activate TLR-4 (Jounai *et al.*, 2012).

In the *in vitro* study, we focused our attention on two important factors in cancer mediated inflammation; these factors are the chemokine CCL2 (MCP-1) and pro-inflammatory cytokine TNF-α. The majority of studies have shown that fatty acids (FAs) have a role in modulating many functions of macrophages including cytokine release, such as tumour necrosis factor-a (TNF-α) (Suganami *et al.*, 2007). TNF-a received considerable attention due to its role in the inflammation process observed in several diseases such as rheumatoid arthritis, atherosclerosis, and obesity (Kelley, 2001; Zhang *et al.*, 2009). Our work revealed that FAs (oleic acid and palmitic 2:1) stimulate

TNF-α production by J774 cells. Previous work shows an increase in the production of TNF-α in culture medium of J774 cells after treatment with 100 µM for 24 hours (de Lima-Salgado *et al.,* 2011). However, the production of this pro-inflammatory cytokine was decreased after treatment with oleic acids, which illustrated the anti-inflammatory properties of oleic acid.

As shown by ELISA, the production of TNF-α was increased in the presence of FAs and cell conditioned medium of B16-F10. However, this production was less than cells treated with fatty acids alone. A previous laboratory –based work (PhD thesis Al-Rayahi, 2017) demonstrated that B16-F10 cell line has the ability to produces IL-10 *in vitro*. Moreover, macrophages simulated IL-10 produced an M2 macrophage-related gene expression profile (Makita *et al.*, 2015). Therefore, it is reasonable to speculate that IL-10 in cell-conditioned medium of B16-F10 would polarise macrophages towards an M2 phenotype. Moreover, production of TNF-α which is regulated by IL-10, is dependent on types of stimulus, which used to activate the macrophage. (Agbanoma *et al.,* 2012).This would explain the decreased production of TNF-α after stimulation with B16-F10 conditioned medium.

Another important player is the chemokine CCL2 (MCP-1) which is one of the main chemoattractant for tissue monocytes/macrophages (Deshmane *et al.,* 2009). Chronic inflammation is a powerful inducer of many types of cancers, and the expression of cytokines/chemokines within the tumours microenvironment (TME) is correlated with poor prognosis (Nicolini *et al.,* 2006). Co-culturing of murine macrophages (J774) with cell-conditioned medium of B16-F10 with FAs resulted in a significant production of CCL2 compared to other conditions (untreated cells, cells treated with FAs or cells co-cultured with conditioned medium from B16-F10). Additionally, the results show that B16-F10 tumour cells produce less of this chemokine. Our results agree with Jung and colleagues (2015) who found that the levels of CCL2 mRNAs in B16F10s were dramatically increased when co-cultured with both macrophages and mature adipose tissue. Moreover, a previous study on the impact of obesity on breast tumour, found that co-culture of the mouse mammary tumour cells (E0771) with macrophages and adipocytes isolated *ex vivo* from obese adipose tissue resulted in the highest production of CCL2 in support of our findings (Santander *et al.,* 2015).

Given the role of CCL2 as a chemokine, it was important to investigate the effects of these conditions on macrophage (J774) migration. Therefore, *in vitro* wound healing assays were performed which revealed that the combined action of cell conditioned medium of B16-F10 and FAs significantly enhanced J774 macrophage migration after 24 hours. Additionally, the results also showed that treatment with 450 µM FAs significantly induce J774 migration compared to both untreated and cells treated with conditioned medium of B16-F10 only. This is may be attributed in part of into the fact that macrophages are highly expressed into specific fatty acids receptor (GPR120) (Shewale *et al.*, 2017). The resent results partially agreed with Jung *et al*. (2015), who found that monocyte migration was moderately increased when B16-F10 cell conditioned medium or mature adipocytes were present, whereas the migration was dramatically increased when the cells were co-cultured with cell B16-F10 conditioned medium, mature adipocytes, and M2-macrophages. GM-CSF and LPS are widely used to generate M1 macrophages *in vitro*, while, M-CSF and IL-4 are used to generate M2 macrophages (Zhang and Kolonin, 2013; Lawrence and Natoli, 2011). In our *in vitro* experiments, we investigated the effect of FAs on macrophage activity without stimulation. Quantitative RT-PCR was done to measure iNOS and Arginase-1 gene expression as canonical markers for M1 and M2 phenotypes respectively. Macrophages exhibit a distinct biomarker profile that can be used to define M1 *vs* M2 cells. M1 macrophages are classically activated by lipopolysaccharide (LPS) and Interferon gamma (IFNγ) to produce high levels of pro-inflammatory cytokines (IL-6, IL-1β, and TNF- α) and nitric oxide (NO). While, exposure to certain cytokines such as IL-4, IL-10, or IL-13 can alternatively activate macrophages into an M2 phenotype with an anti-inflammatory cytokine producer such as (IL-10) and also Arginase -1 (Cheng *et al.*, 2017). Macrophages may enhance or suppress tumour growth and it is associated with the way that macrophages metabolise arginine (Mills, 2001).

Both M1 and M2 cells using arginine in different ways. For example, M2 cells metabolize arginine to ornithine and urea through the Arginase -1 pathway (Mills, 2001). In contrast, M1 macrophages induce inducible NO synthase (iNOS) to produces toxic NO (Ley, 2017). The results show that macrophages co-cultured with conditioned medium containing B16-F10 and FAs for 24 hours have significantly increased iNOS mRNA expression compared to both control cells and cells treated with B16-F10 cell conditioned medium. However, iNOS expression was significantly higher in J774

macrophages co-cultured with FAs for 24 hours. Conversely, the expression of Arginase-1 mRNA was significantly higher in J774 treated with cell-conditioned medium of B16-F10 compared to others. These findings concur with previous laboratory-based work (PhD thesis Al-Rayahi, 2017) who found that Bone Marrow derived macrophages had more Arginase-1 mRNA expression upon B16-F10 stimulation.

3.5 In summary

FAs modulate phenotypic and tumourgenic behaviour of Murine macrophages (J774) and tumour cells (B16-F10) in terms of migration, proliferation, expression of specific receptors and chemokines, cytokines secretion as well as expression of specific polarisation markers of macrophages. Furthermore, HFD derived serum enhanced cell migration and proliferation of mouse skin cancer B16-F10.

Chapter 4

Effect of diet-induced obesity on tumour growth in *LDLR*$^{-/-}$ mouse model

Effect of diet-induced obesity on tumour growth in *LDLR*$^{-/-}$ mouse model

4.1 Introduction

The incidence of individuals being overweight or obese has been rising very rapidly in both developed and developing countries (Jung *et al.*, 2015). Clinical and animal experimental studies have shown that overnutrition, leading to adipose tissue enlargement, increases the risk and progression of several cancers including malignant melanoma (Pandey *et al.*, 2012). However, the mechanistic associations between cancer and obesity are multidimensional and not well established. Chronic inflammation is a well-known mediator of cancer and it is also a feature of obesity (Deng *et al.*, 2016). The chronic inflammatory state in obesity is driven by the excess of non-esterified fatty acids and accounts for activation and chemoattraction of immune cells in those consuming a high fat diet (Divella *et al.*, 2016). Obesity associates with other inflammatory pathologies such as asthma, colitis and, indeed cancers, the focus of the present work (Alvarez-Curto and Milligan, 2016). A growing body of evidence linking obesity and cancer has focused on the systemic inflammatory effects of adiposity, which leads to an increase in the levels of circulating pro-inflammatory adipokines, cytokines, chemokines, insulin, estrogen and other factors directly secreted by obese adipose tissues or indirectly induced to be secreted by other tissues (Fontana *et al.*, 2007). In HFD induced-obesity, alterations of adipokines and cytokines create a chronic inflammatory microenvironment that favours tumour cell motility, invasion, and proliferation (Schmidt *et al.*, 2015). Many of the cytokines correlated with pro-inflammatory states are not only up-regulated during obesity but may also enhance the self-renewal of cancer stem cells. Furthermore, it has been suggested that low grade inflammation can increase the risk of different cancers by providing bioactive molecules from cells infiltrating the tumour microenvironment (TME) including cytokines; growth factors; chemokines that maintain a sustained proliferative rate and cell survival signals (Landskron *et al.*, 2014). Expansion of adipose tissues caused by prolonged consumption of dietary fat is associated with increased tumour infiltration by immune cells including regulatory T cells (T-regs), a critical regulator of the adaptive immune response. Tregs are considered as a major player in tumour immune suppression and

one of the main obstacles to effective anti-tumour immunotherapy (Tang *et al.*, 2011). Taken together, adipocytes, infiltrating immune cells and secreted pro-inflammatory adipokines and cytokines provide a microenvironment favourable for tumour growth (Malvi *et al.*, 2015; Balistreri *et al.*, 2010).

4.2 Aims

An *in vivo* study was designed to investigate the role of diet-induced obesity on melanoma tumour growth, as well as the expression of fatty acid and leptin receptors, cytokine, chemokine profiles, and the modulating effect of obesity on the tumour microenvironment (TME) in an *LDLR*$^{-/-}$ mouse model.

4.3 Animal experimental work

LDLR$^{-/-}$ mice on a C57BL/6J background were bred in a pathogen free barrier facility in groups in ventilated cages at 21^0C, 50% humidity, with 12/12 hours light/dark cycle, and had ad libitum access to food and water. All mice were maintained on 5LF2 (14% protein, 6% fat, 65% carbohydrate), so-called maintenance diet and they were obtained from Jackson Laboratories (B6.129S7-Ldlr$^{tm1Her/}$J). At 6 months' of age, they were randomized to two groups; Group one will be referred to as high fat group fed on a formulated cholesterol free diet 58R3 (high fat) (Test Diet ® product 20% protein, 36% fat, 35% carbohydrate, rich in sucrose) for 2-weeks or 10-weeks prior to tumour implantation .Whereas , the second group of mice were fed on control diet 58R1 (Test Diet ® product 14.8% protein , 4.8 %fat, 73.9 % carbohydrate, low Soybean meal) . Body weights were measured weekly, and then both groups were injected subcutaneously with 5×10^5 B16-F10 cells and monitored daily for the presence of palpable tumour. Once tumours were established they were measured daily and when they reached to the endpoint, usually after 14 days, mice were sacrificed, and serum was collected from heart puncture, tumours and spleen were collected for analysis (**Figure 4-1**).

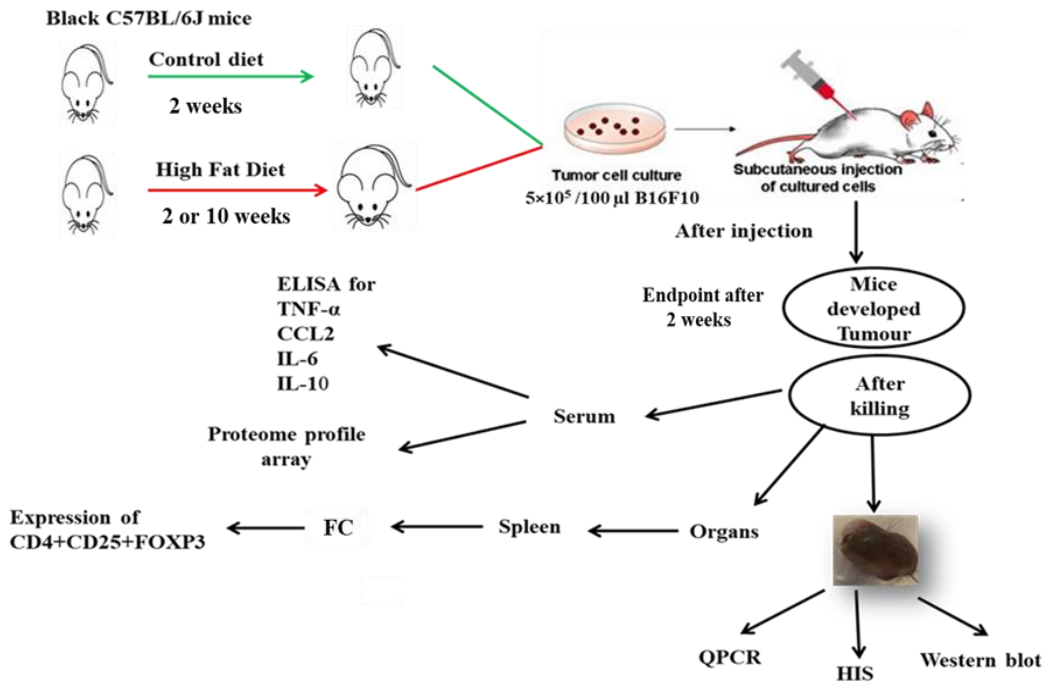


Figure 4-1: Schematic of *in vivo* experimental design

4.4 Results

The impact of 4- and 12-weeks' high fat diet feeding on melanoma tumour growth and progression in *LDLR*$^{-/-}$ mouse model was studied.

4.4.1 The effect of a 4-week fat rich diet on tumour growth

8 *LDLR*$^{-/-}$ mice (4 Females and 4 males) were given a high fat diet (58R3), and 9 (4 females and 5 males) were given a low-fat diet (58R1).

4.4.1.1 The effect of a 4-week fat rich diet on body weight gain

The extent of body weight increase was determined in 6 months old mice fed either control low- fat, (CD) (58R1) or high fat diets (HFD) (58R3) for 2-weeks prior to B16-F10 injection and then another 2-week during the tumour bearing period. All mice were sex and age matched and given equal amounts of either diet. Their body weight was measured weekly (**Figure 4-1**). The increase in weight gain percentage was calculated according to this formula:

(Final body weight / initial body weight ×100) -100.

Weekly monitoring revealed that mice fed on a low-fat diet (58R1) had little body weight gain or remained stable (**Figure 4-2A**). However, mice fed on the high fat diet had a greater body weight gain after 4 weeks feeding (**Figure 4-2B**).

Furthermore, after 4-weeks of feeding, body weights of HFD group were significantly increased compared with the control diet (p= 0.0195) (**Figure 4-3A**). The male group fed on HFD showed significant increase in their body weight percentage compared to their counterparts fed on CD (p= 0.0137) (**Figure 4-3C**). However, no significant differences were observed amongst females of both groups (p=0.4261) (**Figure 4-3B**). The photos in figure (**Figure 4-4B**), showed the accumulation of abdominal fat in mice fed on diet rich in fat compared to less or near absence of fat in mice fed on low-fat diet (**Figure 4-4A**). Our results clearly showed that feeding fatty diet for 4-weeks significantly increased the weight gain compared to control diet feeding; however, this effect was higher but not significant in females. The results also indicate the differences in obtaining weight gain between sexes, hence, the males fed on HFD for 4-weeks showed more susceptible to gain weight compared to females fed on the same diet (**Figure 4-2B** and **C**).

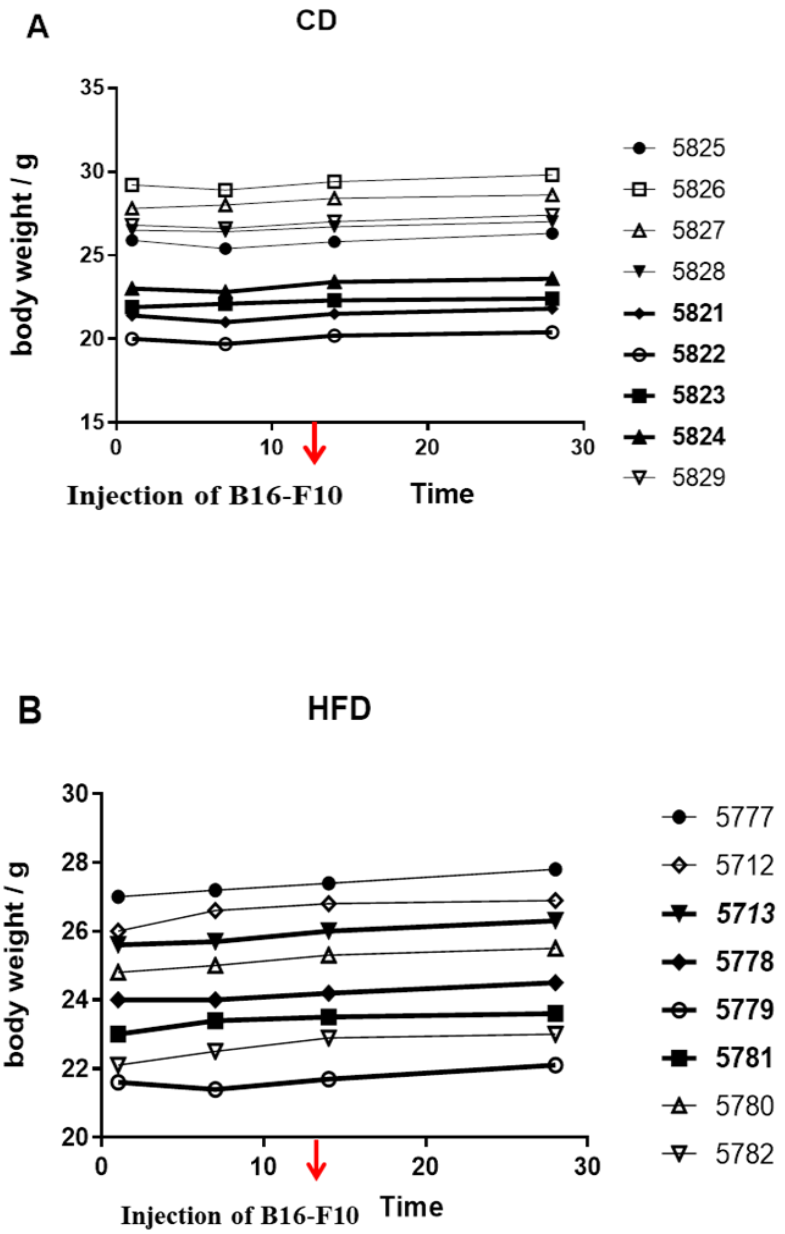


Figure 4-2: Weekly weight gain of *LDLR*$^{-/-}$ tumour bearing mice after 4-weeks feeding with either a CD or a **HFD**

(**A**) Body weight gain over 4-week observation period of CD feeding. While, (**B**) Changes in body weight of mice fed on high fat diet for the same period. Bold font refers to females while the light font to males.

* The numbers represent the ID number of each mouse, and numbers in bold font refer to females.

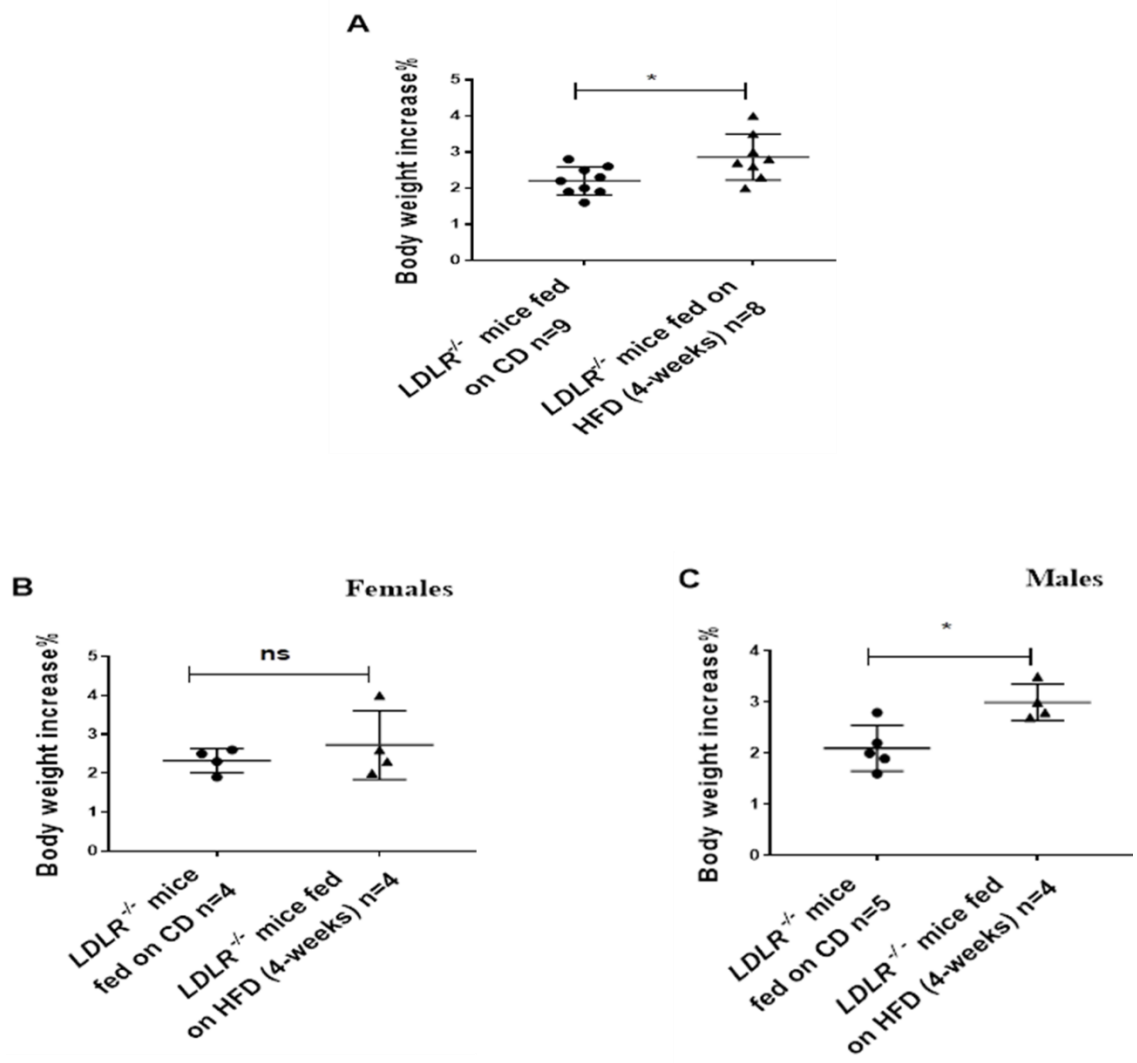


Figure 4-3: Measurments of body weight in *LDLR*$^{-/-}$ tumour bearing mice following 4-weeks feeding HFD or CD

(**A**) Percentage of weight gain observed after 4-weeks feeding HFD and CD. Body weight increased in female and male groups fed on HFD and CD diets (**B** and **C**). Data are presented as means as means ± SD.and statistical analysis was performed by unpaired *t* test. ($^{*}p$ <0.05).

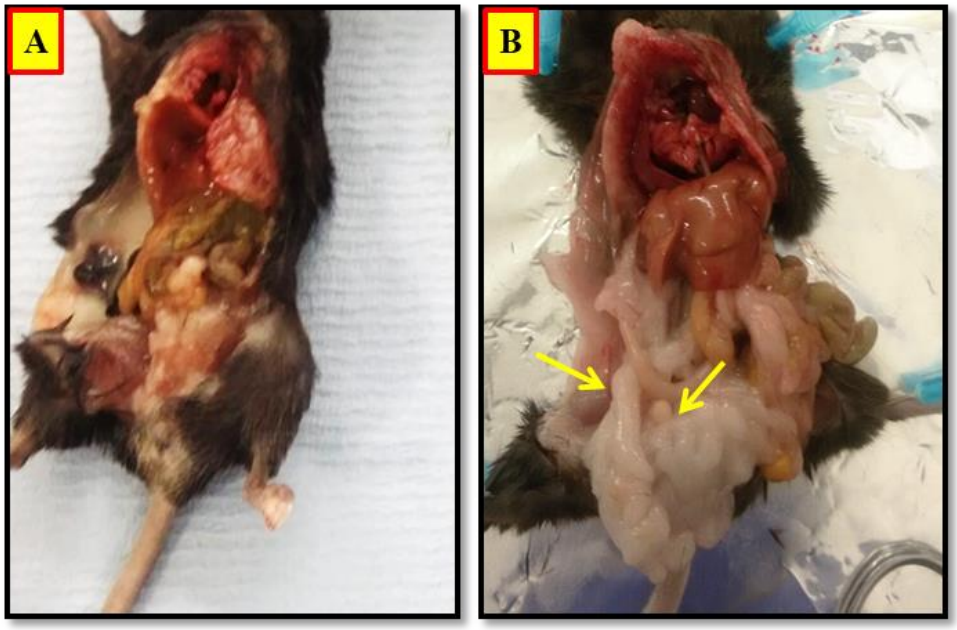


Figure 4-4: Representative images of *LDLR*$^{-/-}$ tumour bearing mice after 4-weeks feeding CD or HFD.
(**A**) Picture shows no fat deposition in control mice was notice. (**B**) Photo shows increased in body size and yellow arrows indicate expansion of abdominal fat after 4-weeks consuming diet rich in fat.

4.4.1.2 Dietary rich in fat increases non-esterified fatty acids (NEFA)

Accumulating evidence showed that high fat diet induced -obesity is associated with chronic elevation of saturated fatty acids (FAs) and development of inflammatory changes (Boden, 2008). Fatty acids play an important role in the development of obesity-related metabolic complications (Jung and Choi, 2014). Excessive release of fatty acids (FAs) from expanded adipose tissues can activate Toll-like Receptor (TLR-4) and may lead to increase nuclear factor kappa B-dependent (NF-*κB*) expression and upregulate the expression of pro-inflammatory genes, including TNF-α, IL-1β and thereby promote tumour growth in mouse models of obesity. Furthermore, a metabolic advantage is superseded by the activity of these and other pro-inflammatory cytokines that are thought to act in a manner akin to wound healing (Iyengar *et al.*, 2013). Therefore, the levels of non-esterified fatty acids (NEFA) as important mediators in high fat diet–induced obesity in *LDLR*$^{-/-}$ tumour bearing mice were determined in experiments that examined whether consumption of a HFD for 4-weeks affects the levels of NEFA. NEFA levels were significantly higher in sera of *LDLR*$^{-/-}$ tumour bearing mice fed on HFD (58R3) compared to those fed on low fat diet (58R1)

(p=0.0020) (**Figure 4-5A**). NEFA levels were slightly higher in males group fed on HFD (58R3) compared to females fed on the same diet (200.3 ± 6.043 *vs* 209.5 ± 6.335) (**Figure 4-5B and C**). However, both groups had significantly higher NEFA levels in contrast to their counterparts fed CD (58R1) (**Figure 4-5B and C**). These results indicate that consuming diets rich in fat are significantly associated with increased non-esterified fatty acid in the circulation.

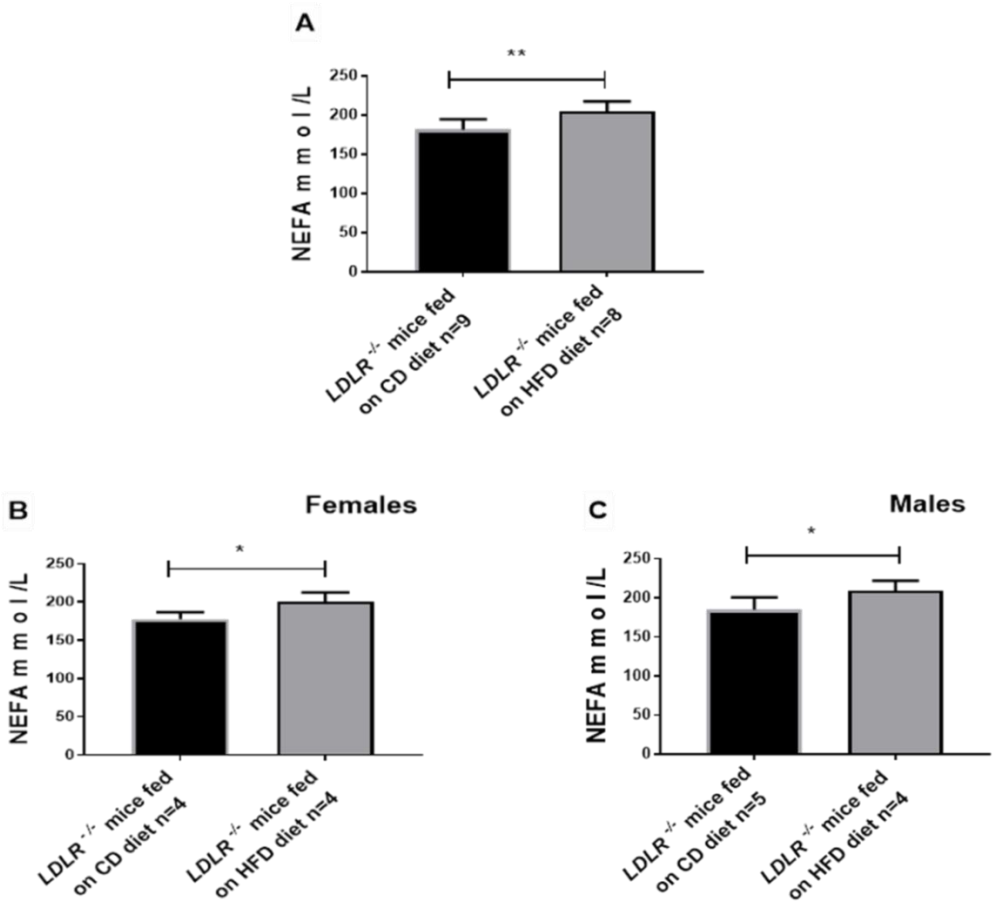


Figure 4-5: Effect of 4-weeks consumption of a diet rich in fat on the NEFA levels in *LDLR*$^{-/-}$ tumour bearing mice.

(**A**) Quantitative measurements of NEFA in serum of *LDLR*$^{-/-}$ tumour bearing mice fed on a HFD or CD in male and female mice. (**B**) Female and (**C**) male *LDLR*$^{-/-}$ tumour bearing mice fed on HFD or CD for 4-weeks. The data are presented as means ±SEM from three independent experiments and each sample was measured in triplicate n=(3). Statistical analysis was performed by unpaired *t* test. (*p <0.05).

*One mouse from control diet group was excluded from further analysis due to ectopic injection.

4.4.1.3 Characterisation of syngeneic melanoma tumour implanted in *LDLR*$^{-/-}$ mice fed on high fat diet and control diet

4.4.1.3.1 Macroscopic examination of tumours

Macroscopic examination shows melanotic tumour masses in the subcutis. Tumours were circumscribed and nodular. Some melanoma tumours dissected from mice fed on HFD were embedded in thick adipose tissue layer (**Figure 4-6B**), compared to less or absent fat surrounding tumours in mice fed on CD (**Figure 4-6A**). There was no evidence of melanotic lymph nodes and no obvious necrosis of tumour masses. The site of injection was visually checked for infiltration of tumour and vessel formation after extracting the tumour masses. Tumour weight and volume were measured.

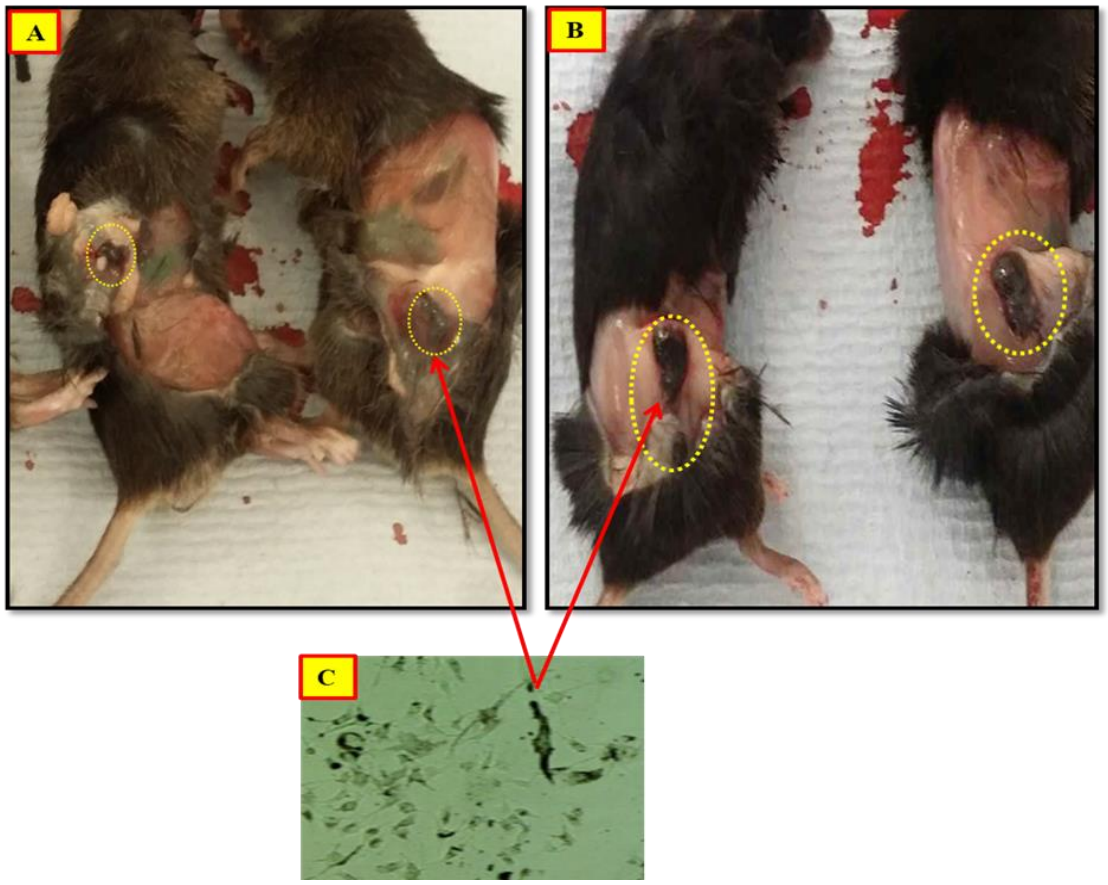


Figure 4-6: Representative images of melanoma tumour dissected from *LDLR*$^{-/-}$ male mice fed on a HFD or CD.
(**A**) Subcutaneous melanoma tumours in *LDLR*$^{-/-}$ mice fed on CD. (**B**) Increased in tumour sizeer after 4-weeks of a HFD feeding.

4.4.1.3.2 The effect of 4-weeks feeding dietary fat on tumour volume

This experiment compared the tumour volume in *LDLR*$^{-/-}$ mice fed HFD or CD for 4-weeks to investigate the effect of dietary consumption rich in fat on tumour size. All tumours were measured *ex vivo* upon excision. Tumours extracted from mice fed on a high fat diet were significantly (p= 0.0160) larger than those found in mice from the low-fat diet group (**Figure 4-7A**). When breaking down the groups according to sexes the female group fed on HFD had larger tumour volumes compared to control diet fed mice (160.5 ± 12.76 *vs* 139 ± 8.803) (**Figure 4-7B**). The results also showed that males bearing significantly bigger tumours compared to their counterpart fed on CD (p= 0.0103) (**Figure 4-7C**).

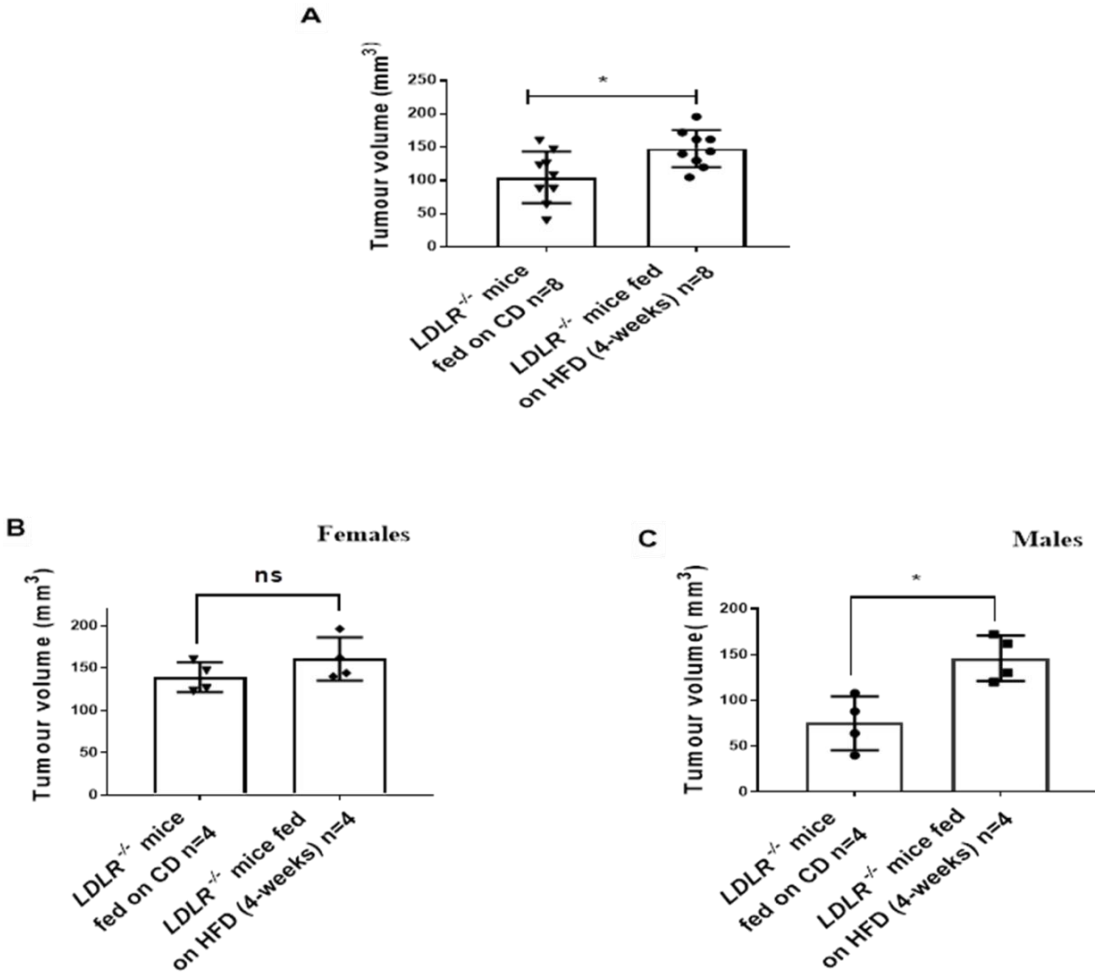


Figure 4-7: Tumour volume after a 4-week feeding a HFD in *LDLR*$^{-/-}$ tumour bearing mice. (**A**) Tumour volume in all ***LDLR***$^{-/-}$ mice fed a HFD or a CD. (**B**) The effect of consuming a HFD on tumour volume in female and (**C**) Male mice subgroups. The data are presented as means ± SD and statistical analysis was performed by unpaired *t* test ($^{*}p$ <0.05).

4.4.1.3.3 The effect of a diet rich in fat on melanoma tumour weight

The data showed a significant increased in body weight gain after 4-weeks feeding high fat diet compared to CD group (in male mice). Therefore, the influence of HFD- induced obesity (58R3) on melanoma tumour weight was examined after 4-weeks of feeding and compared to a low -fat diet (58R1). Tumours in both high fat diet and control diet were dissected after 10-14 days after syngeneic B16-F10 injection. Although there were noticeable differences in the time of initiation of tumour formation between the two groups, tumours progressed rapidly in the HFD group compared to the CD. The results show that tumour weight in HFD group was significantly greater than tumours in the CD group (p= 0.0101) (**Figure 4-8A**). Furthermore, the data also showed that that male mice from the HFD group had a significant increase in tumour weight compared to CD (p=0.0235) (**Figure 4-8C)**. However, no significant differences were found in tumour weight in females fed on a HFD compared with those fed on CD (**Figure 4-8B)**. These results were consistent with an increase in weight gain after consuming dietary fat.

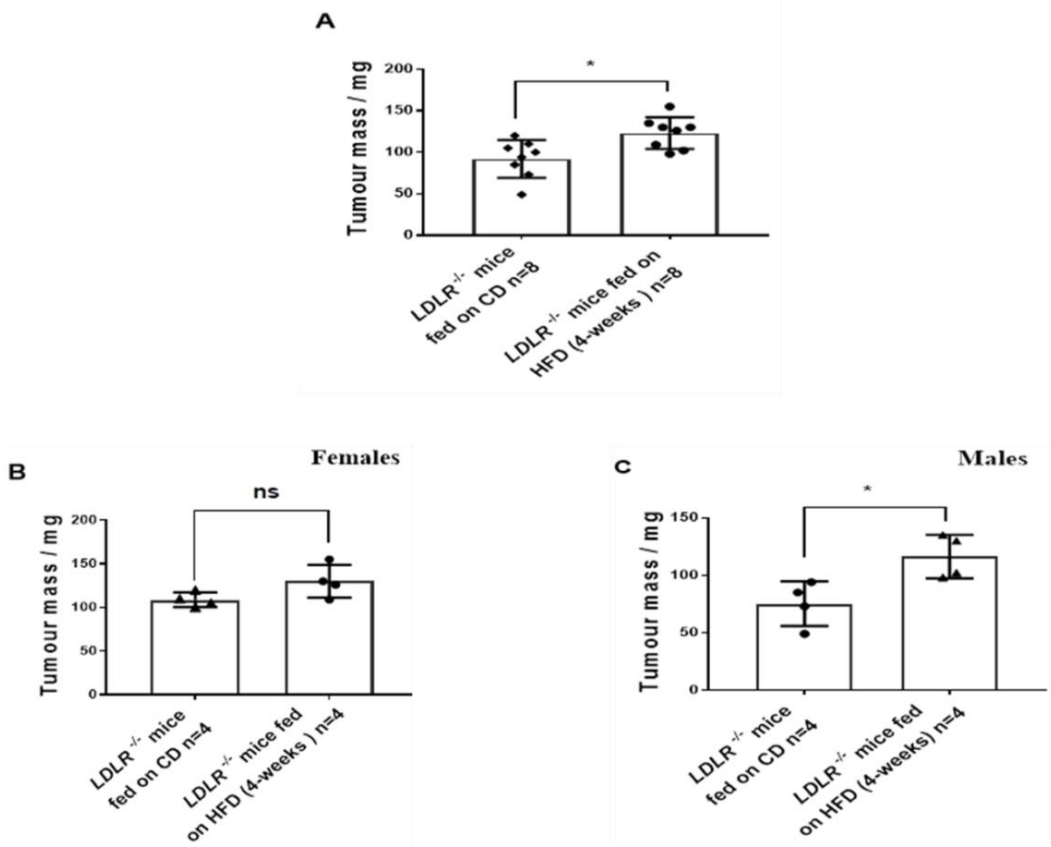
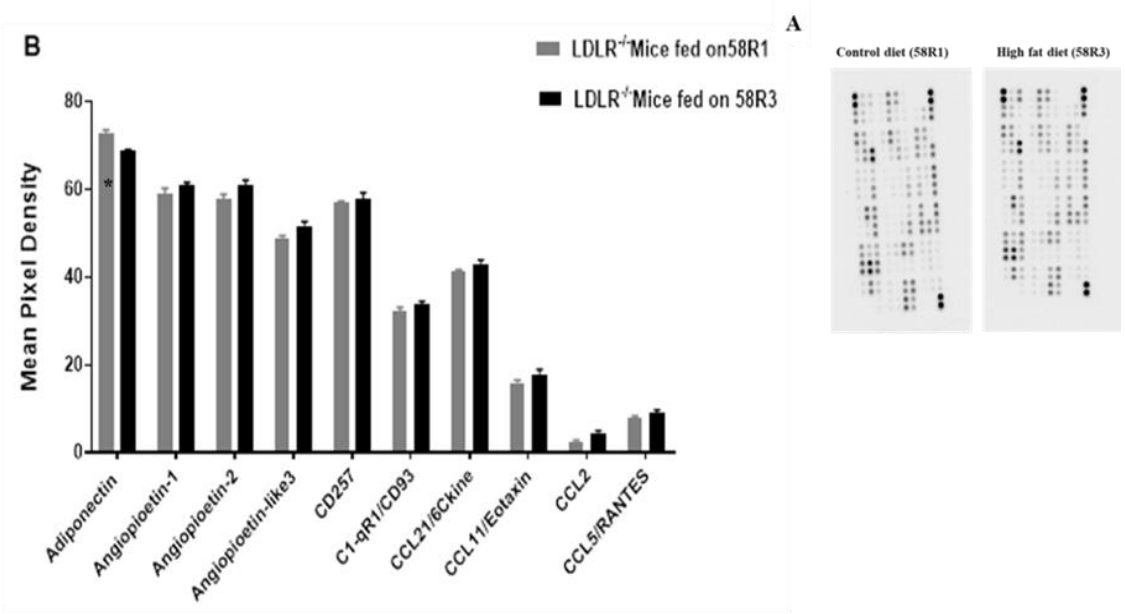


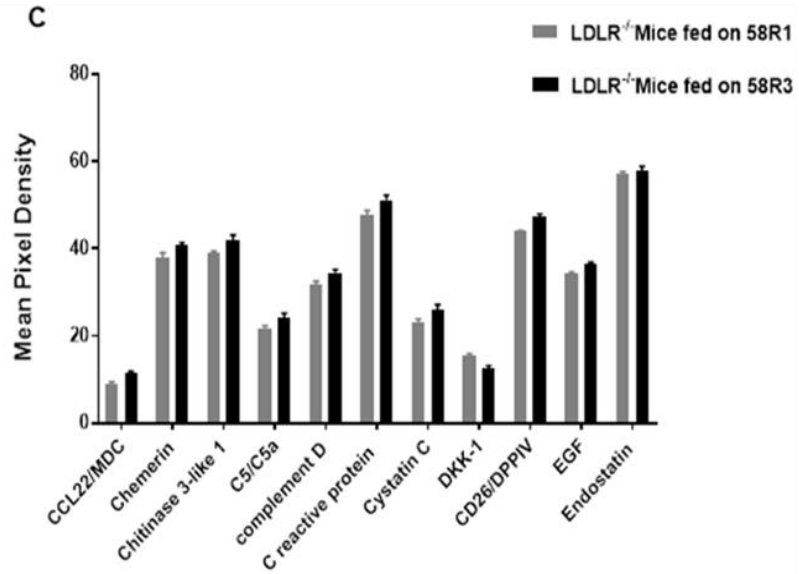
Figure 4-8: Effect of 4-weeks feeding a HFD on tumour weight in *LDLR*$^{-/-}$ tumour bearing mice.

(**A**) Effect of dietary fat on tumour weight in both genders. Panel (**B** and **C**) Tumour wieght of females and males mice fed on a CD and a HFD. The data are presented as means ± SD and statistical analysis was performed by unpaired t test. ($^{*}p < 0.05$).

4.4.1.4 Dietary rich in fat induces changes in cytokine profiles

Cytokines have a significant role in cancer-related inflammation with a direct and indirect impact on tumour growth and development (Amedei *et al.*, 2013; Landskron *et al.*, 2014). Therefore, the main purpose to perform proteome array was to determine the effect of high fat diet after 4-weeks feeding on cytokines production and to deduce their relationship to melanoma tumour growth. There was a significant increase in the adipokine Leptin (p=0.0106) and Resistin (p=0.0321) in HFD groups compared to control groups in contrast to Adiponectin which is found to be significantly (p=0.0122) elevated in CD compared to HFD group. The changes in these three cytokines mirror the metabolic adaptation in response to a HFD. Further, the results also indicate that fatty diet consumption induced an increase in the production of distinct growth factors, adhesion molecules, chemokines and cytokines compared to control diet (**Figure 4-9**). Not all mediators tested responded to the stimulation. Those that did are indicated with an asterisk and will be discussed further.


A
Control diet (58R1)
High fat diet (58R3)
B
LDLR-/- Mice fed on58R1
LDLR-/- Mice fed on 58R3
Mean Pixel Density
80
60
40
20
0
*
Adiponectin
Angiopioetin-1
Angiopioetin-2
Angiopioetin-like3
CD257
C1-qR1/CD93
CCL21/6Ckine
CCL11/Eotaxin
CCL2
CCL5/RANTES


C
LDLR-/- Mice fed on 58R1
LDLR-/- Mice fed on 58R3
Mean Pixel Density
80
60
40
20
0
CCL22/MDC
Chemerin
Chitinase 3-like 1
C5/C5a
complement D
C reactive protein
Cystatin C
DKK-1
CD26/DPPIV
EGF
Endostatin

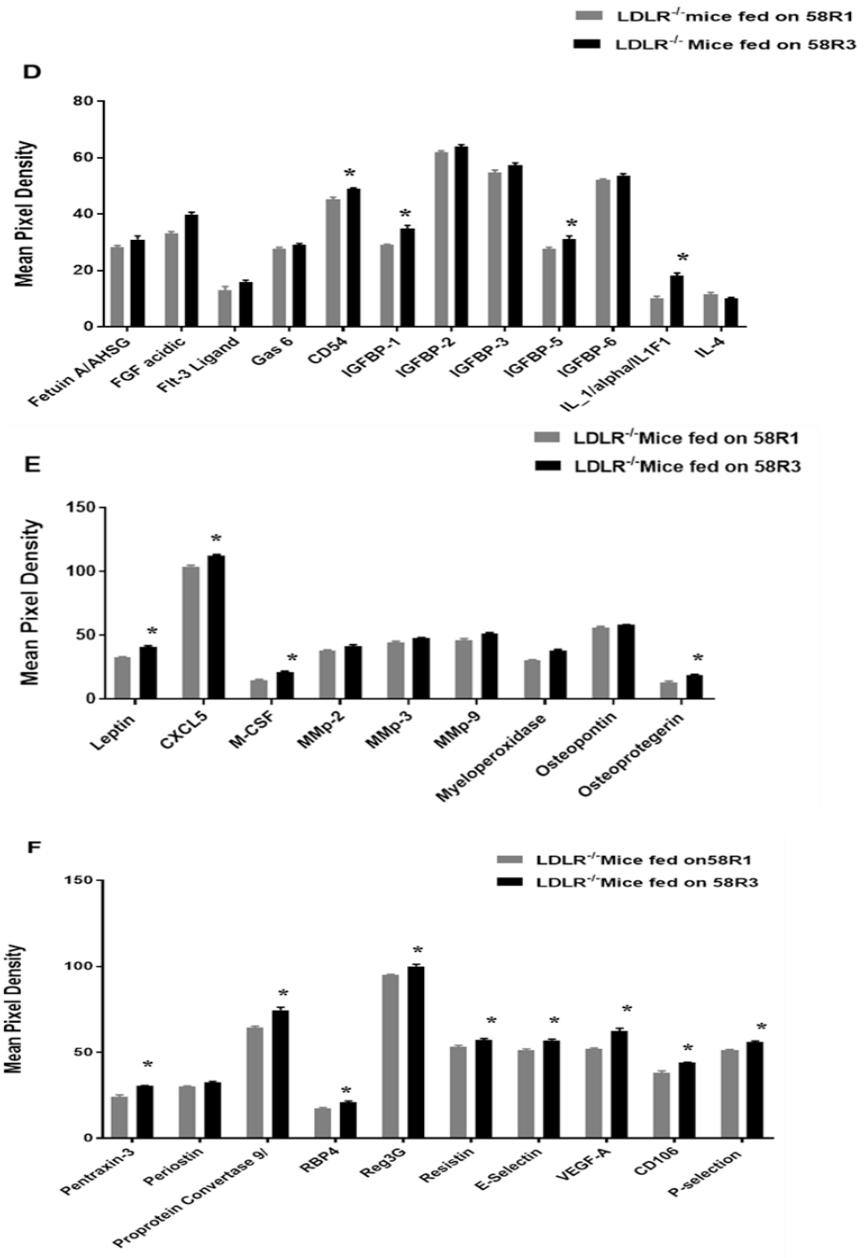


Figure 4-9: Effect of HFD feeding on inflammatory mediaters and rescipter in sera of females *LDLR*$^{-/-}$ tumour bearing mice.

(**A**) Membrane array and the data from a five minutes exposure to film. (**B-F**) Identified cytokines and chemokine by the array. The data are presented as means ±SEM and statistical analysis was performed by Two-way ANOVA /Sidak's multiple comparisons test. (*$p < 0.05$). The degree of differences determined by the statistical tests was confirmed to represent the original specific intensities by independent assessors. Cytokines and chemokines that did not express in the blots were:
CCL21/6,CXCL13/BLC/BCA/,ENDOGLIN/CD105,IFNGAMMA,IL33, LIPOCALIN, LIX,IL-27,IL-28,IL-33,CCL3,CCL4,MIP-1,CXCL9/MIC,CXCL10/IP-10,alpha/beta,CCL6/10-CCL17/TARC,CL19/MIP-3beta, CCL20/MIP-3alpha,CD40/TNFRSF5,CXCL2/MIP-2,CXCL1-1/I-TAC,CXCL13/BLC/BCA,CXCL16, Endostatin, FGF-21, G-CSF,GDF-15, HGF, ICAM-1/CD54,IL-1beta/IL-1F2,TNF-alpha, kine,CD14,CD40/TNFRSF5, CD160,Cogulationfactors - CX3CL1,CCL12/MCP-5,IL-10, Fractalkine.

4.4.1.5 Analysis of Splenocytes T-regulatory cells in *LDLR*$^{-/-}$ tumour bearing and *naïve* mice fed on a HFD

The percentage of T-regs in spleen of *LDLR*$^{-/-}$ tumour bearing and tumour free mice (*naïve* mice) fed on a HFD was investigated using flow cytometry analysis. The purpose behind this experiment was to give evidence that there is an increase of T-reg cells in mice bearing tumour. The results showed that mice bearing tumours had significantly higher T-regs percentage in their spleen compered to naïve mice fed a HFD (p= 0.0001) **(Figure 4-10A**). The results also showed that both sexes bearing tumour exhibit significant increase in T-regs percentage compared to tumour free mice (**Figure 4-10B and C**). To distinguish T-regs population, three markers ($CD4^+CD25^+FOXP3^+$) were used (**Figure 4-11**).

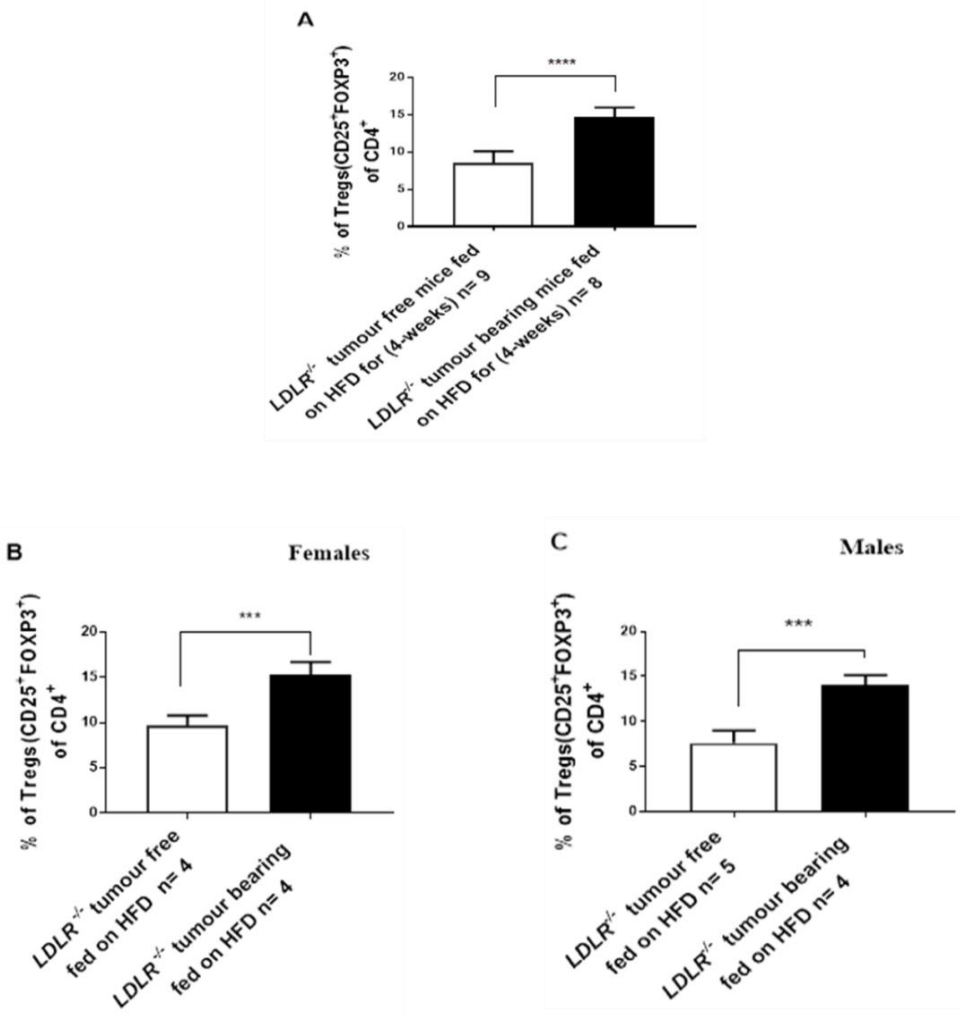


Figure 4-10: Flow cytometry analysis for T-regs population in *LDLR*$^{-/-}$ tumour bearing mice and tumour free mice fed on HFD for 4-weeks.

(**A**) Effect of dietary fat on the percentage of splenic T-regs population in both groups, while (**B** and **C**) Percentage of T-regs in female and male groups, respectively. The data are presented as means ±SEM and the statistical analysis was performed by unpaired *t* test. (*$p < 0.05$).

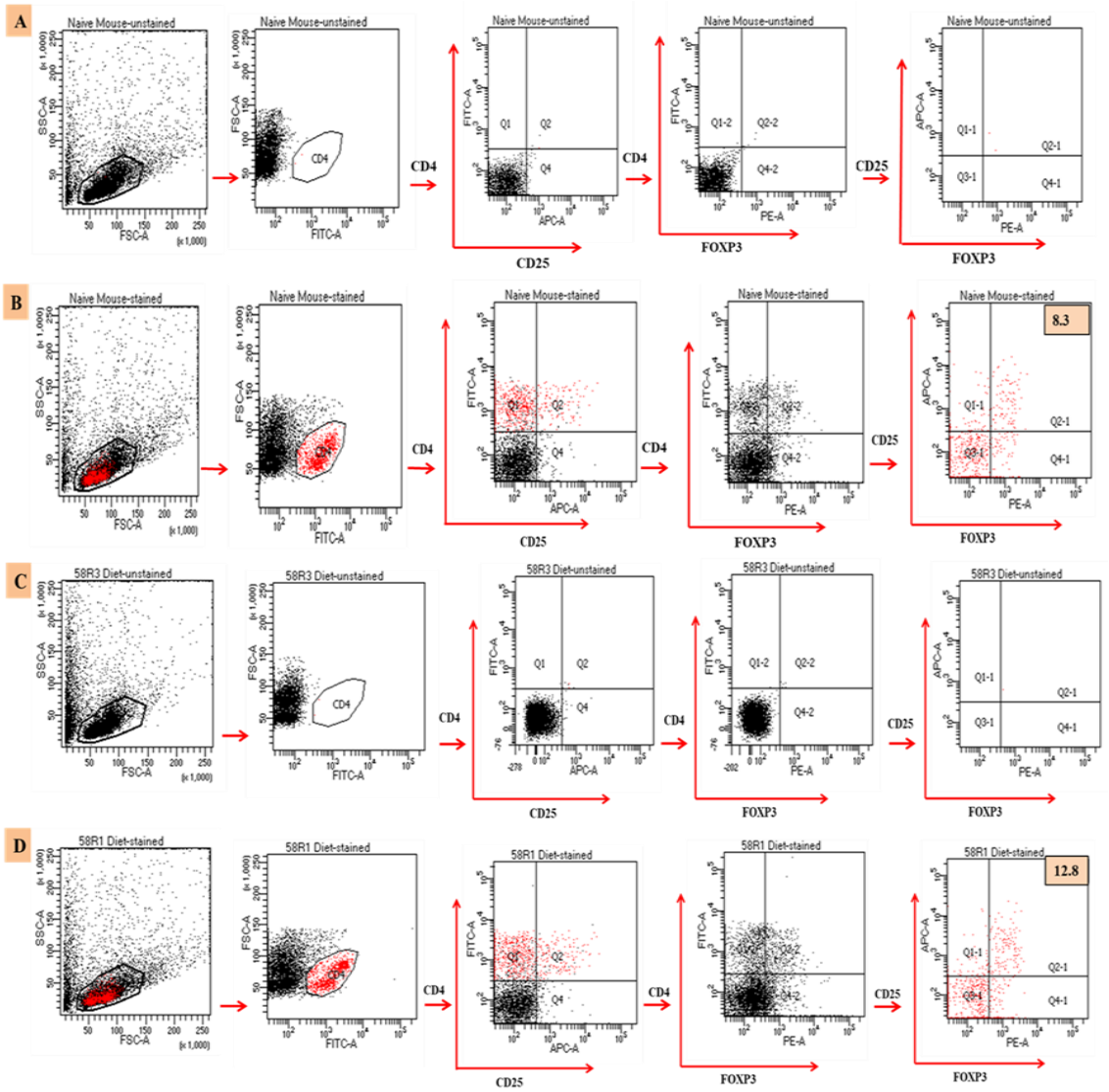


Figure 4-11: Representative staining of splenocytes cells of *LDLR*$^{-/-}$ tumour bearing mice and *Naïve* (tumour free) mice after 4-weeks feeding HFD.

Three markers were used to identify T-regs populations by flow cytometry analysis CD25-APC, CD4- FITC and FOXP3-PE. The gating was done on CD4^{+} cells and the percentage of T-regs was calculated using, BD FACS Diva TM software version 8.0 runs on the Microsoft Windows 7. (**A** and **C**) Dot plots graphs for unstained spleen suspension cell of *LDLR*$^{-/-}$ tumour free and tumour bearing mice fed on HFD (58R3) diet, respectively. (**B** and **D**) Dot plots graphs for stained spleen suspension cells of *LDLR*$^{-/-}$ tumour free and bearing mice fed on HFD (58R3) diet, respectively. Numbers in the plots indicate the percentage of Tregs from gating CD4^{+} cells.

4.4.1.6 The impact of a 4-week HFD on T-regulatory accumulation in spleen of *LDLR*$^{-/-}$ tumour bearing mice

Thus, this experiment aimed to investigate the effect of diet rich in fat on splenic T-regs in tumour bearing mice FC revealed that spleens of tumour bearing mice fed on a high fat diet had a significant increase in the percentage of T-regs compared to those fed on low fat diet (p= 0.0111) (**Figure 4-12A**)**.** However, when analysing by gender, no significant difference was observed in T-reg among splenocytes in female mice (13.7 ± 0.4491 *vs* 15.25 ± 0.71*)* (*p*= 0.1146) (**Figure 4-12B**), but there was a significant difference in male mice fed a HFD (p=0.0153) (**Figure 4-12C**). Flow cytometry results clearly demonstrated the modulatory effect of fatty diet on tumour microenvironment after 4-weeks, which was consistent with the increase in body weight gain of male mice and enhancement of tumour growth. Moreover, it is apparent that the splenocytes of female mice fed on HFD contained slightly more T-regs in relation with female mice fed on CD; nevertheless, it was not significant (**Figure 4-12B**). Therefore, the effect of long term consumption of a diet rich in fat was investigated.

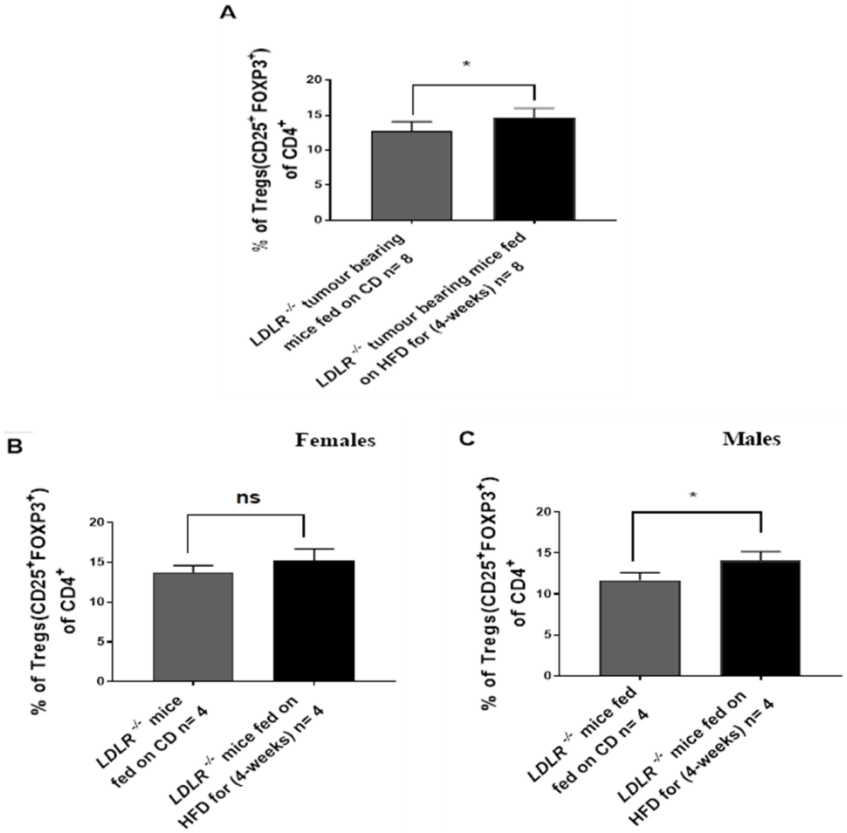


Figure 4-12: Analysis of T-regs population in *LDLR*$^{-/-}$ tumour bearing mice after 4-weeks feeding CD or HFD by Flow cytometry.

(**A**) The effect of fatty diet on the percentage of splenic T-regs population in both groups, while (**B** and **C**) this percentage in female and male groups, respectively. FACS analysis was carried

out using three markers to identify T-regs population ($CD4^+CD25^+FOXP3^+$). The data are presented as means ±SEM and statistical analysis was performed by unpaired *t* test. ($^*p < 0.05$).

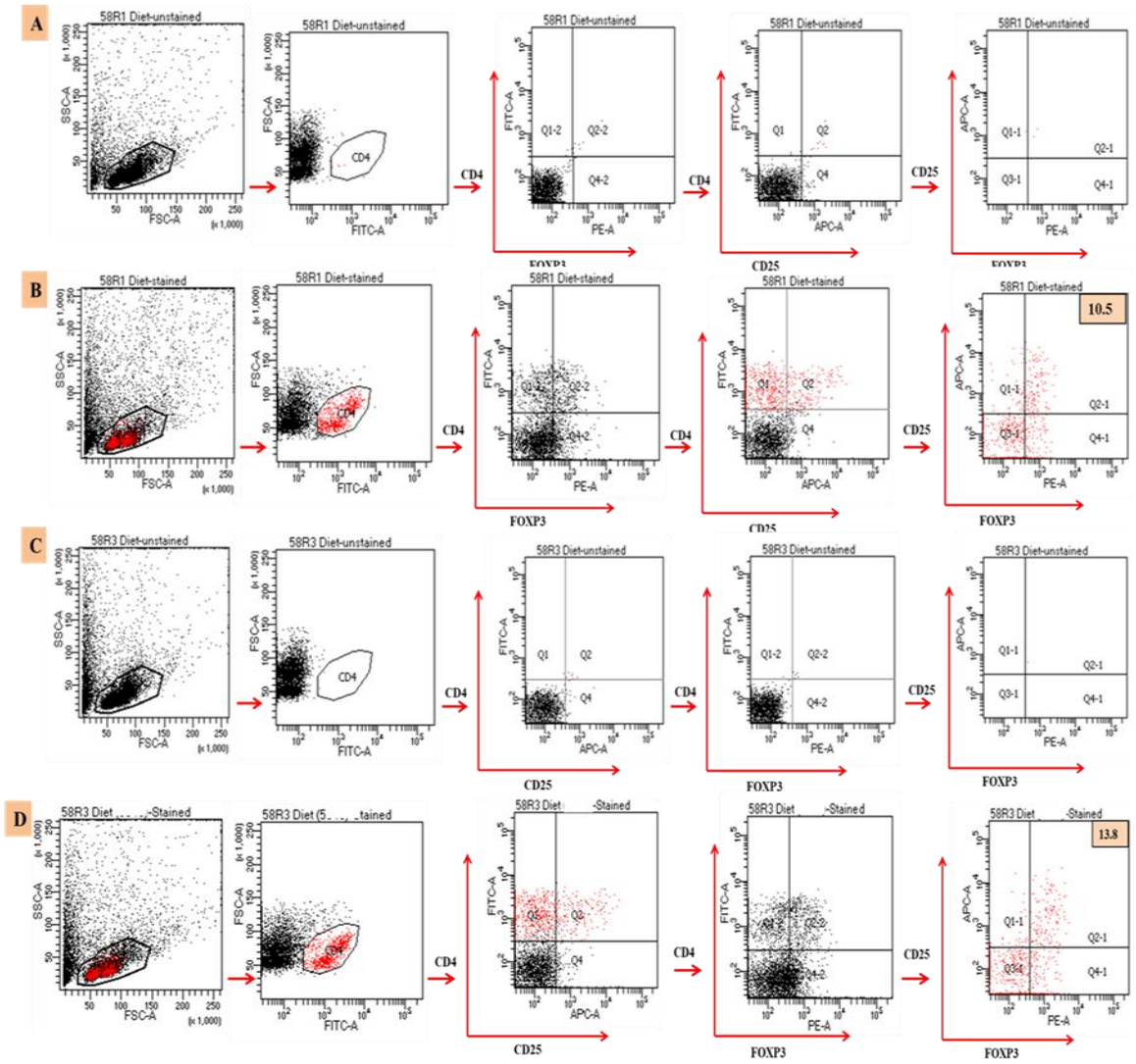


Figure 4-13:Representative dot plots showing the % of T-regulatory($CD4^+CD25^+FOXP3^+$) population in the spleen of *LDLR*$^{-/-}$ tumour bearing mice after 4-weeks feeding a HFD or a CD

FC analysis was done using three markers; CD25-APC, CD4-FITC and FOXP3-PE to distinguish the Treg population. The gating was done on $CD4^+$ cells and the percentage of Tregs was calculated using, BD FACS Diva TM. (**A** and **C**) dot plots graphs for unstained spleen suspension cell of *LDLR*$^{-/-}$ tumour bearing mice fed on CD and HFD diet, respectively. (**B** and **D**) dot plots graphs for stained spleen suspension cell of *LDLR*$^{-/-}$ tumour bearing mice fed mice fed on 58R1 and 58R3 diet respectively. Numbers in the plots indicate the percent of gated $CD4^+$ cells.

4.4.2 The effect of a 12-week HFD feeding on tumour growth

Previous data found an increase in both body weight and tumour weight in the males group after feeding a HFD (58R3) for 4-weeks. However, these observations also showed that tumour growth and weight gain were different between sexes. Therefore, we hypothesized that increasing the length of the HFD to 10-weeks prior tumour injection would significantly increase the weight gain in the animals and would further enhance melanoma tumour growth compared to CD. A previous laboratory-based work (PhD thesis Kheder,2017) who found that 10-weeks duration of the HFD induced histological changes consistent with non-alcoholic fatty liver disease (NAFLD) and increased inflammatory markers.

4.4.2.1 Chronic consumption of HFD induces significant increase in weight gain

To investigate whether long term consumption of a HFD induced significant changes in weight gain compared to control. *LDLR*$^{-/-}$ mice (4 Females and 5 males) were given low- fat diet (58R1) and 8 mice (4 females and 4 males) were fed the HFD (58R3) for 10 weeks prior to tumour implantation. The body weight of each mouse was measured weekly to examine the effect of the HFD. After 12-weeks feeding diet rich in fat both male and female mice had increase body weight (**Figure 4-14**). Moreover, the difference in weight change was significantly greater in the HFD group and continued to increase over time (p<0.0001) (**Figure 4-15A**). The female and male groups fed on a HFD were also heavier compared to their CD littermates (**Figure 4-15B and C**). Additionally, macroscopic examination revealed an increase in subcutaneous fat, mesenteric fat, and retroperitoneal fat in mice fed on the HFD for 12-weeks (**Figure 4-16C and D**) compared to the CD. Furthermore, all animals showed reduce in fat deposition (**Figure 4-16A and B**).

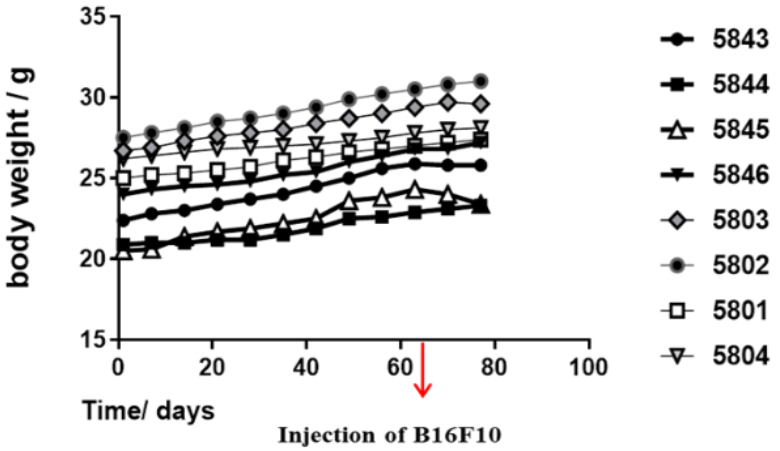


Figure 4-14: Changes in the body weight after (12-weeks) feeding a HFD.
LDLR$^{-/-}$ mice consumed a diet rich in fat for 12- weeks post tumour implantation. After 14 days, tumours and spleens were dissected and analysed *ex vivo* from the animals. The changes in body weight were monitored and measured weekly.

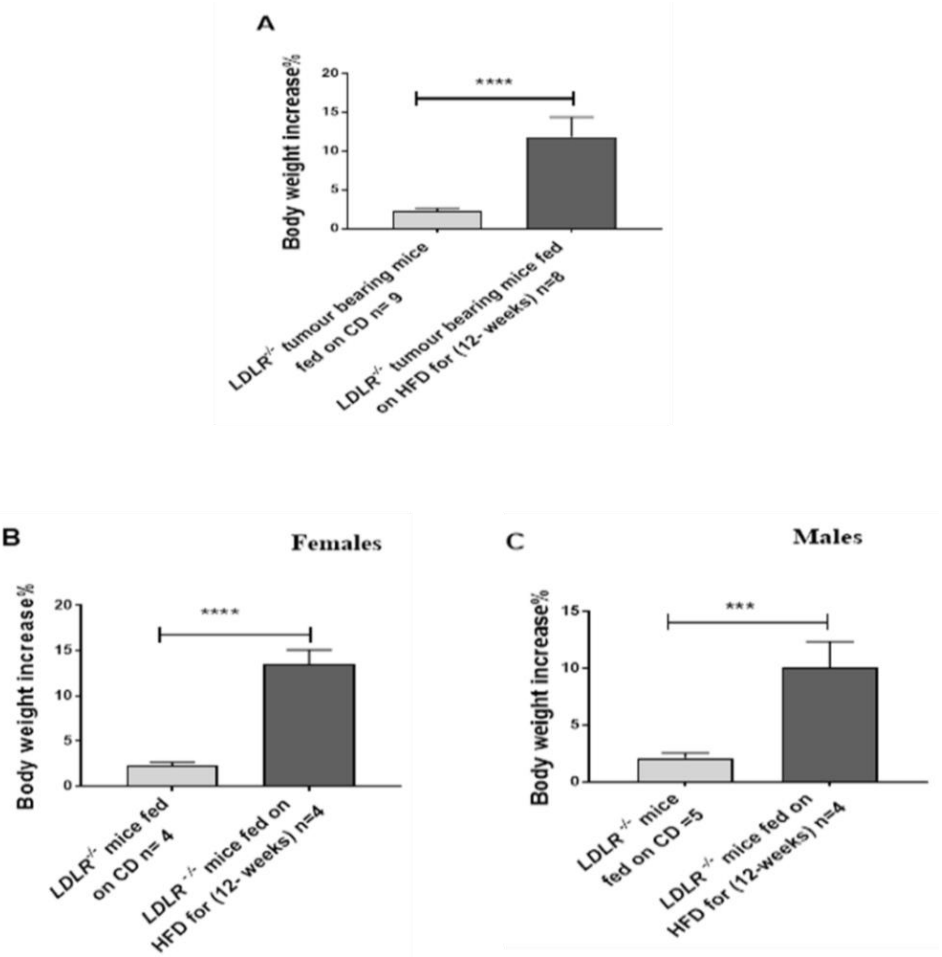


Figure 4-15: Effect of long term (12-weeks) feeding of a HFD on body weight.
(**A**) The body weight of *LDLR*$^{-/-}$ tumour mice after 12-weeks on HFD and CD in all animals. (**B**) The body weight of females and (**C**) males fed CD and HFD subgroup. The data are presented as means ±SD. Statistical analysis was performed by unpaired *t* test ($^{*}p< 0.05$).

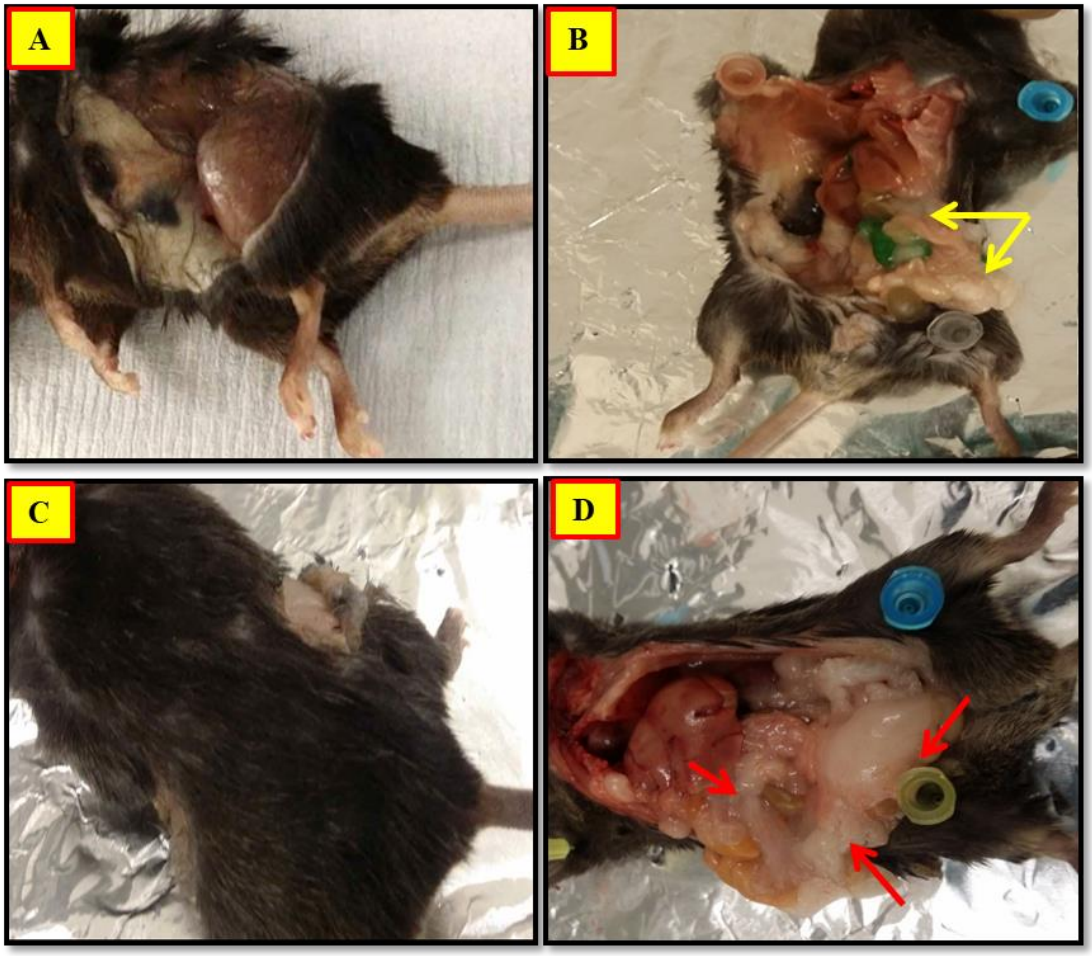


Figure 4-16: Representative pictures of *LDLR*$^{-/-}$ C57BL/6J mice feeding on CD or HFD.
(A) The body size of mice fed on a CD and **(B)** the yellow arrows show reduced or no fat deposition in mice fed on CD **(C)** Increased in body size after 12-weeks consuming HFD and **(D)** red arrows mark the adipose tissue deposit.

* The green colour in gut comes from diet colour which was used as an indicator to help animal technicians when they change the diet weekly.

4.4.2.2 Analysis of tumour burden in *LDLR*$^{-/-}$ mice fed a HFD for 12-weeks

4.4.2.2.1 Phenotypic examination of tumour mass

All tumours have the same morphological characterization as described previously. However, *LDLR*$^{-/-}$ mice that consumed a HFD for 12-weeks developed larger subcutaneous melanocytic tumours compared to both tumours implanted in mice fed on CD or a HFD for 4-weeks. Furthermore, macroscopic examination also showed that these tumours were embedded in the extremely thick layer of fat (**Figure 4-17C and D**) compared to the CD group (**Figure 4-17A and B**).

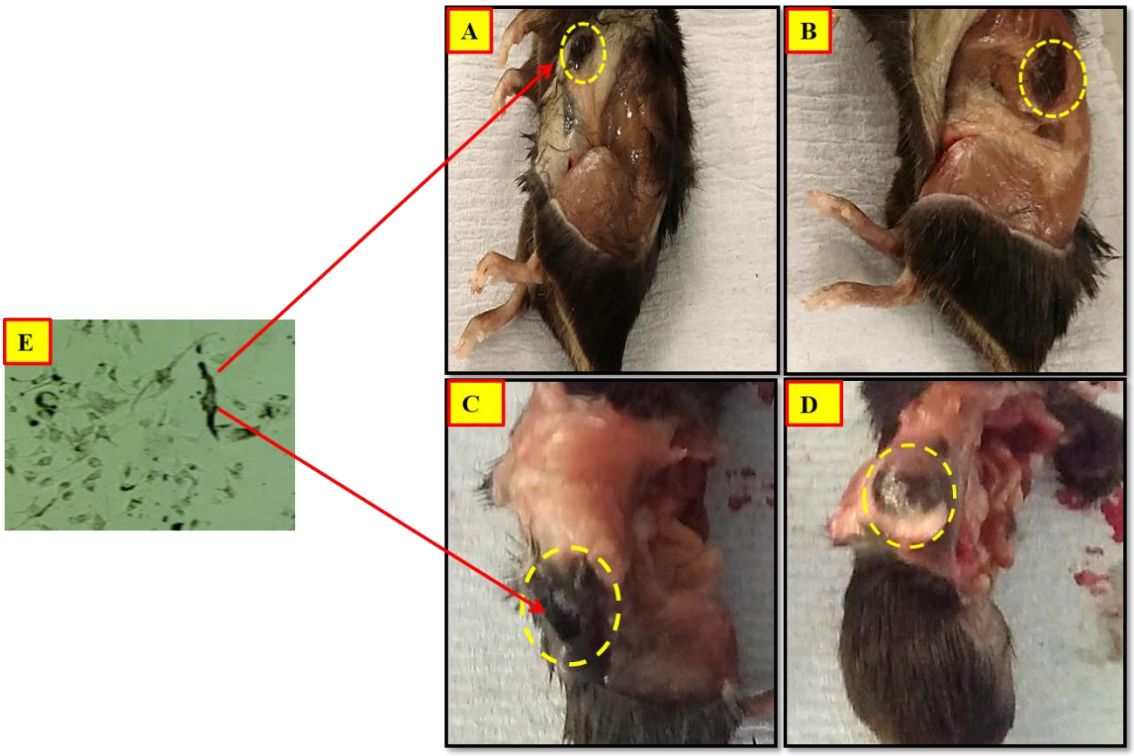


Figure 4-17: Representative pictures of subcutaneous melanoma tumours in *LDLR*$^{-/-}$ mice. (**A** and **B**) Syngeneic melanoma tumours in *LDLR*$^{-/-}$ C57BL/6J background mice fed on a low-fat diet. (**C** and **D**) Increased in tumour size as indicated in yellow circle in mice fed on HFD for 12-weeks. (**E**) Murine melanoma cancer cells (B16-F10).

4.4.2.2.2 Histological characterisation of tumour mass from *LDLR*$^{-/-}$ mice on high fat diet and control diet

The aim of the experiment was to characterise the histopathology of melanoma tumours dissected from *LDLR*$^{-/-}$ mice fed a HFD or CD. Sections were prepared from tumour, stained with haematoxylin/eosin, and analysed microscopically.

Parts of tumour of 6 months-old male *LDLR*$^{-/-}$ mice were fixed and paraffin embedded. Then 4µm slides were prepared, and stained with haematoxylin, and eosin staining. Four LDLR$^{-/-}$ mice fed on HFD for 12-weeks were compared with 4 mice fed on CD. Histological analysis of these tumours reveals masses constituted of pigmented melanoma parenchymal cells that intermingle with multiple other cell types and extracellular matrix components (**Figure 4-18A and B**). Neovascularization of the tumour masses is also observed in both sections however, they were larger and more distributed in tumour section of mice fed on HFD (**Figure 4-18B**), compared to CD (**Figure 4-18A**). The infiltration of fat cells was bigger in size in tumour sections of mice fed on HFD compared to those fed on CD.

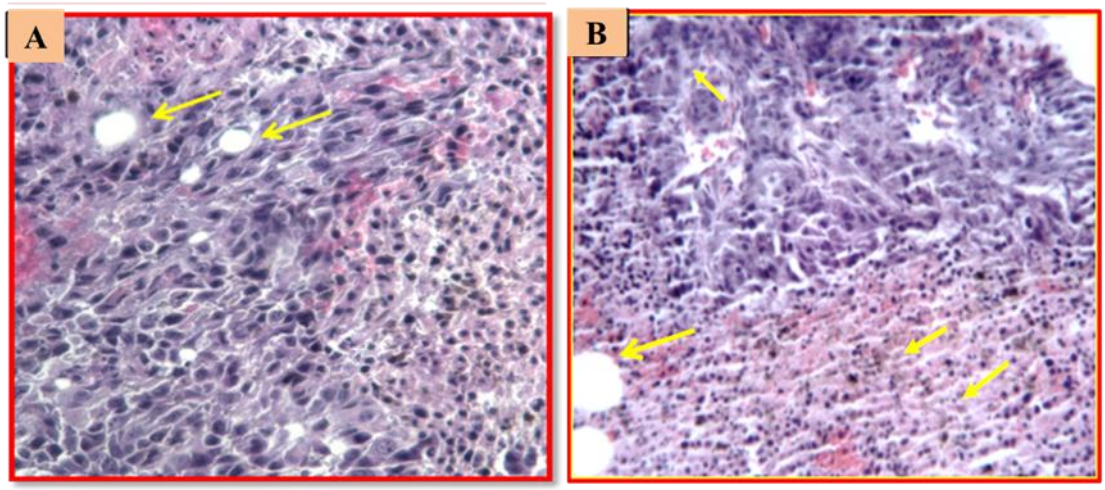


Figure 4-18: Haematoxylin and eosin-stained melanoma-tumour sections from *LDLR*$^{-/-}$ tumour bearing mice fed on HFD and CD.
The Representative images show tumour cells with abundant melanin pigment. **(A** and **B)** Tumour sections of mice fed on CD and HFD, respectively, yellow arrows indicate fat cells in tumour sections and they were bigger in size in tumour section of HFD (Magnification 20x).

4.4.2.2.3 Prolonged consumption of a HFD accelerates tumour volume

This experiment investigated the effect of a 12-week HFD feeding on tumour volume. The data in figure **(Figure 4-19A),** revealed that there was a significantly increased tumour volume (p= <0.0001), and this was accompanied with an increased in body weight gain. Both females and males fed a HFD had a significantly greater tumour volume compared to those fed a CD (p= 0.0001) (**Figure 4-19B and C**).

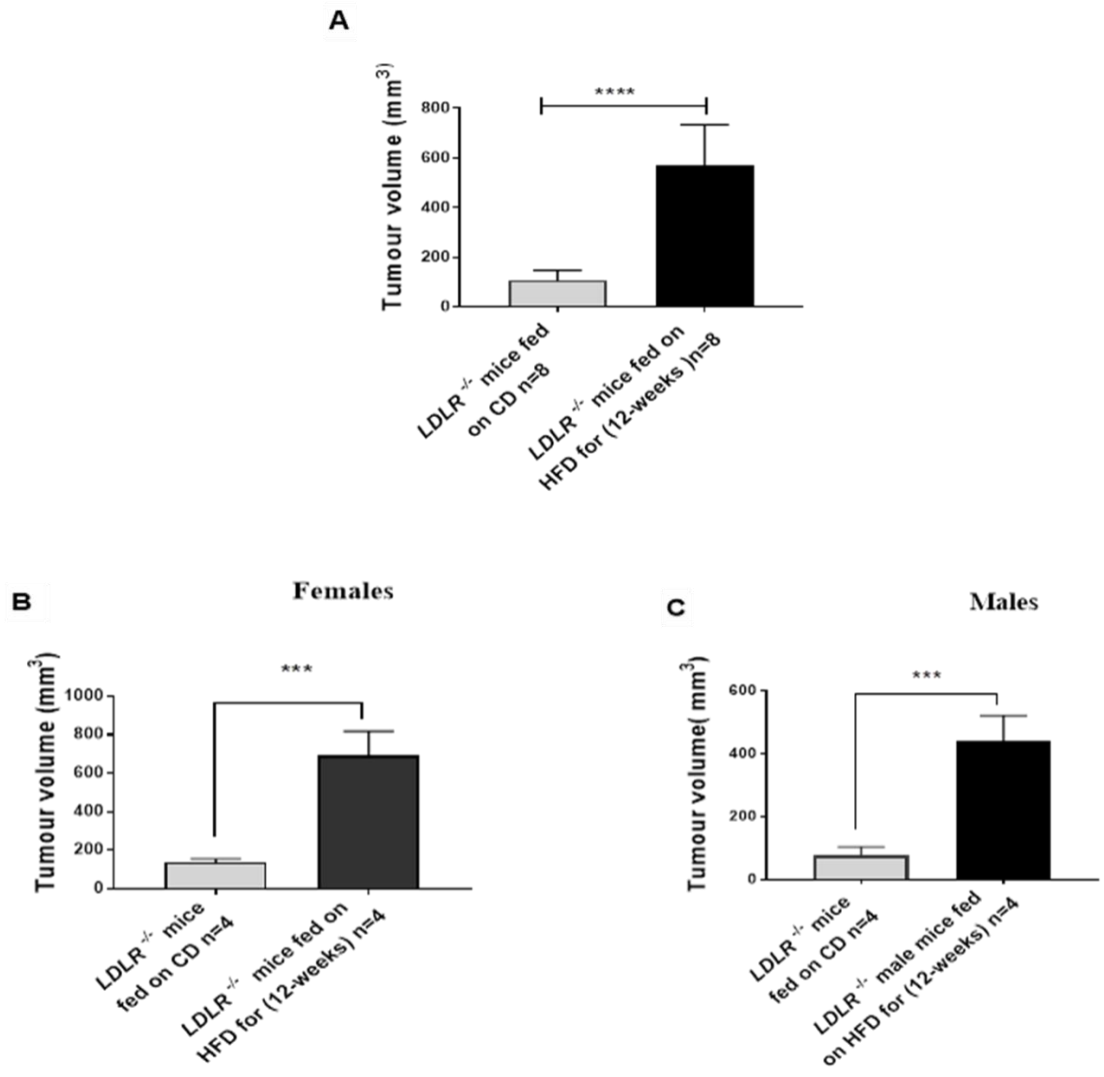


Figure 4-19: Tumour volume in *LDLR*$^{-/-}$ tumour bearing mice fed on a HFD or a CD for 12-weeks.

(**A**) Effect of chronic consumption fatty diet on tumour volume on both groups. (**B** and **C**) Effect on sex (females and males), respectively. The data are presented as means ± SD and statistical analysis was performed by unpaired *t* test. ($^{*}p < 0.05$).

4.4.2.2.4 Long term HFD feeding increases melanoma tumour weight

LDLR$^{-/-}$ C57BL/6J background mice were fed a HFD for 12-weeks (diets were continued until tumour collection) and control diet as described previously. HFD fed mice had increased melanoma tumour weight compared to those fed on CD (**Figure 4-20A**). Furthermore, females fed on a HFD also had significantly higher tumour weight compared to CD fed female mice (**Figure 4-20B**). The results also showed that males fed on HFD bearing significantly heavier tumour compared to their counterpart fed on control diet (**Figure 4-20C**).The findings clearly indicate that obese mice had larger tumours than lean mice because of their HFD.

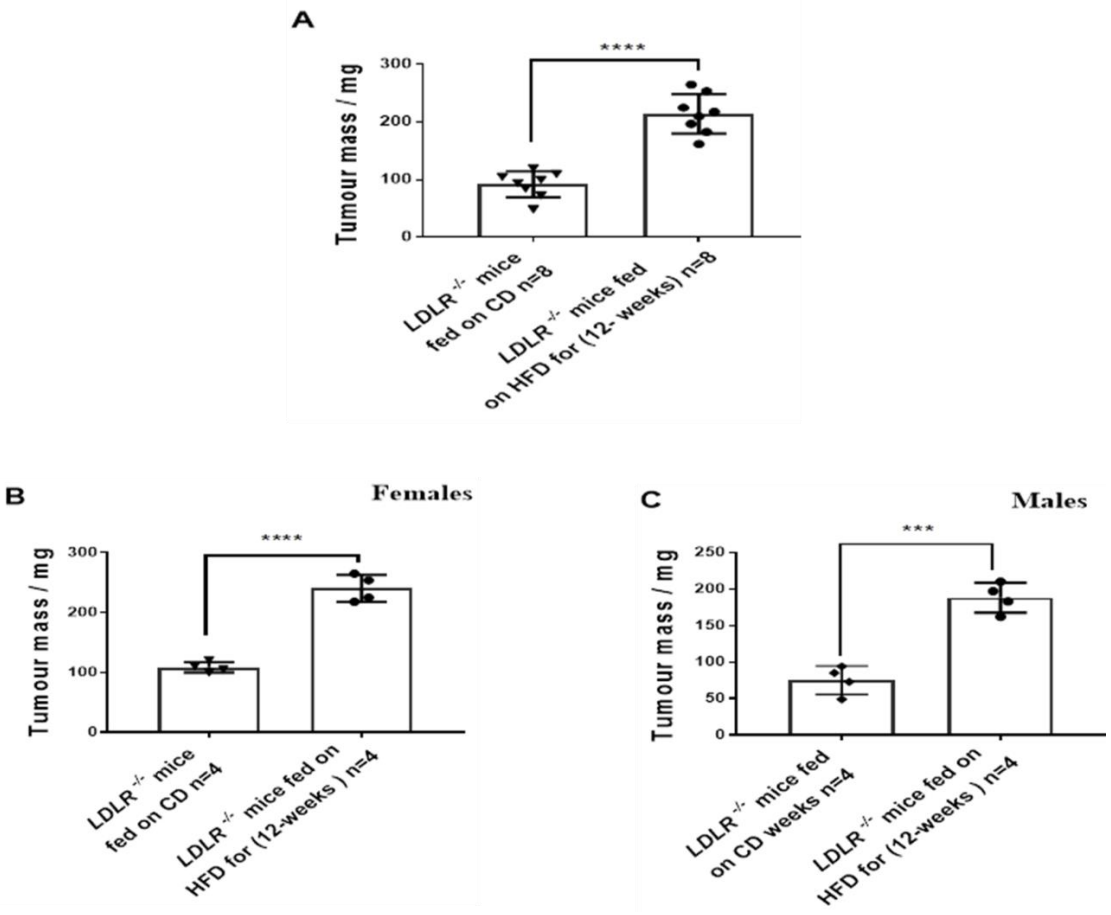


Figure 4-20: Effect of chronic fatty diet consumption on tumour weight in *LDLR*$^{-/-}$ tumour bearing mice.

(**A**) The effect of a HFD on tumour weight in both genders. (**B**) The tumour weight of females fed on CD or HFD diet. (**C**) The effect of HFD on tumour weight in males. The data are presented as means ± SD and statistical analysis was performed by unpaired *t* test. ($^{*}p < 0.05$).

4.4.2.3 Chronic fatty diet consumption increases T-regulatory accumulation in the spleen of *LDLR*$^{-/-}$ tumour bearing mice

FC was done by using lineage markers to identify T-regs ($CD4^+$ $CD25^+$ $FOXP3^+$) **(Figure 4-22**), in splenocytes from *LDLR*$^{-/-}$ tumour bearing mice fed on a HFD (58R3) or CD (58R1) for 12-weeks. There was significant increase in the T-regs population after consuming a HFD compared to those fed CD (**Figure 4-21A**). The results also showed significant increase in the proportion of T-regs in all animals fed a HFD (**Figure 4-21B and C**).

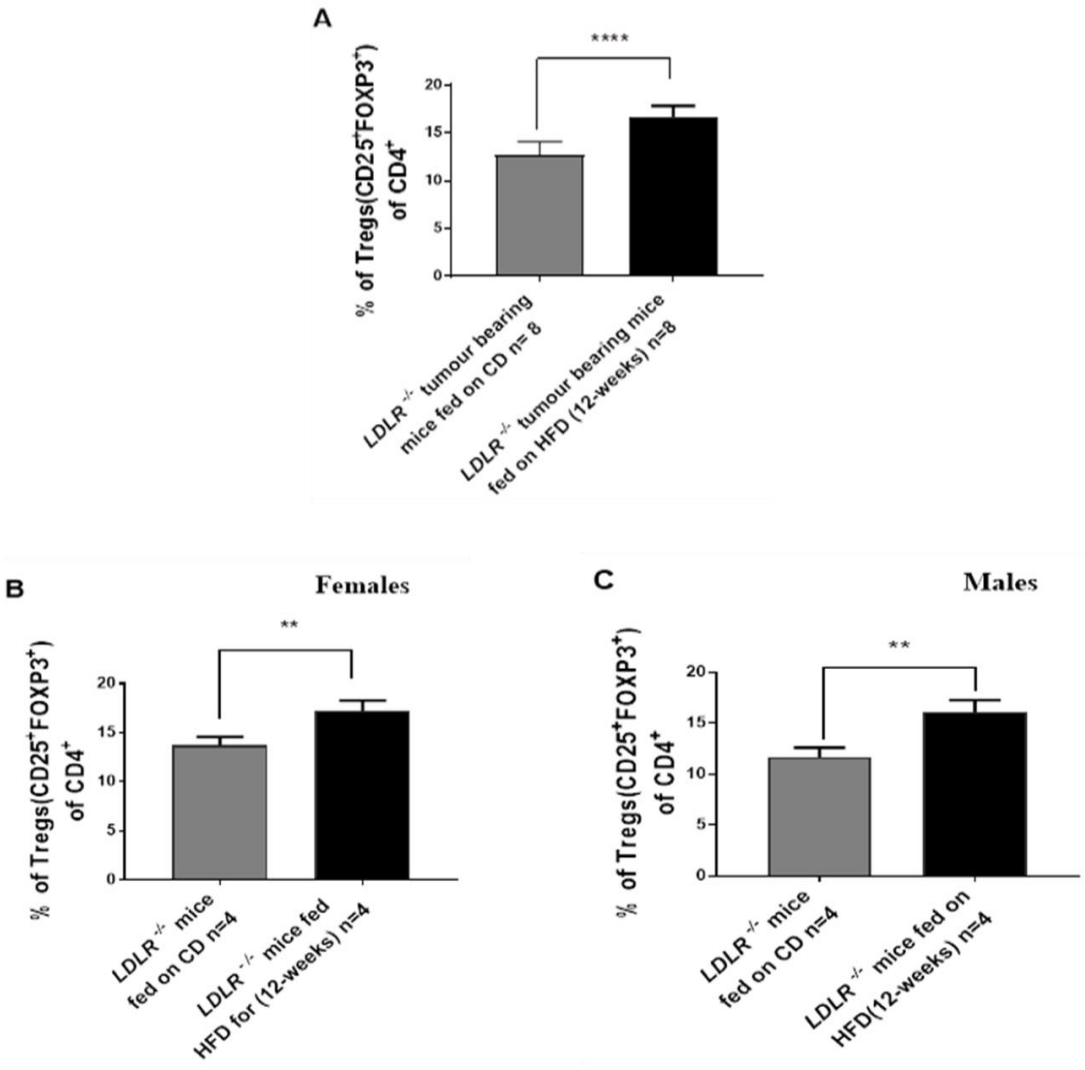


Figure 4-21: Flow cytometry detection of T-regs population in the spleen of *LDLR*$^{-/-}$ tumour bearing mice fed a HFD for 12-weeks.

(**A**) The FC analysis for the percentage of splenic T-regs population in both groups, while panels (**B** and **C**) These percentages in females and males group, respectively. Three markers were used to distinguish T-regs population ($CD4^+CD25^+FOXP3^+$). Data are presented as means ±SEM. Statistical analysis was performed by unpaired *t* test. ($^*p < 0.05$).

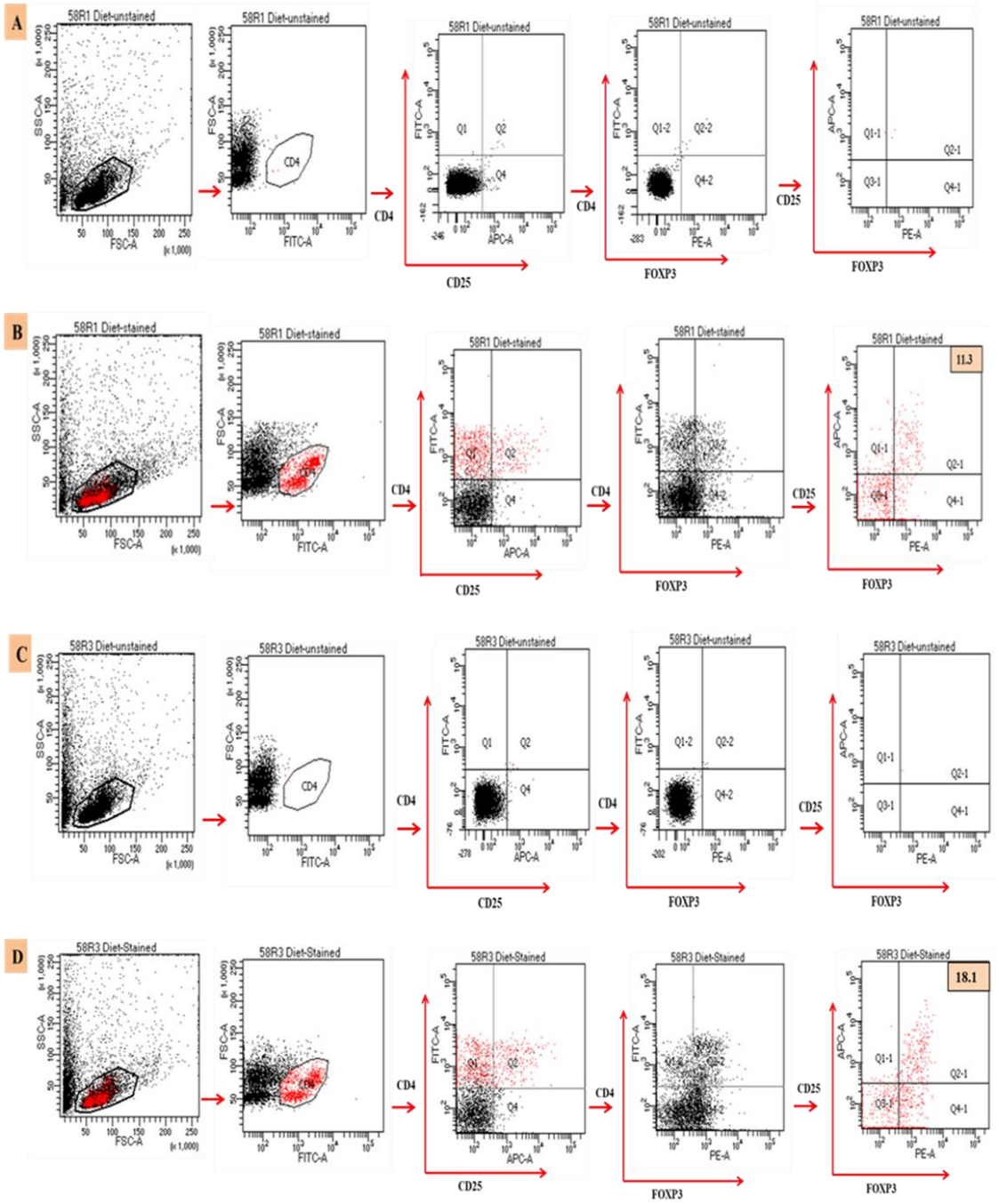


Figure 4-22: Representative dot plots showing the percentage of T-regulatory ($CD25^+CD4^+FOXP3^+$) population in the spleen of *LDLR*$^{-/-}$ tumour bearing mice after 12 weeks feeding HFD or CD

Three markers were used to distinguish T-regs population ($CD25^+$ $CD4^+FOXP3^+$) by flow cytometry (FC). Cells were gated on the $CD4^+$ population. (**A** and **C**) dot plots for unstained splenocytes of *LDLR*$^{-/-}$ tumour bearing mice fed on CD (58R1) and HFD (58R3) diet respectively. (**B** and **D**) dot plots representing stained splenocytes from *LDLR*$^{-/-}$ tumour bearing mice fed mice fed on CD (58R1) and HFD (58R3) diet, respectively. Numbers in the plots indicate the percent of $CD4^+$ gated cells.

4.4.2.4 Analysis of pro-inflammatory markers

4.4.2.4.1 High Fat Diet increases IL-6 levels in *LDLR*$^{-/-}$ tumour bearing mice sera

The levels of IL-6 protein in sera of *LDLR*$^{-/-}$ tumour bearing mice that consumed a HFD for 12-weeks was examined and compared with mice fed on a CD. There was significant increase in IL-6 protein in tumour bearing mice fed on the HFD compared to those fed on CD (p= 0.0001) (**Figure 4-23A**). In male *LDLR*$^{-/-}$ mice fed a HFD, IL-6 protein was significantly higher compared to those fed a CD (p= 0.0032) (**Figure 4-23C**). Furthermore, female mice had a significant increase in the IL-6 levels compared to those fed a CD (p= 0.0098) (**Figure 4-23B**).

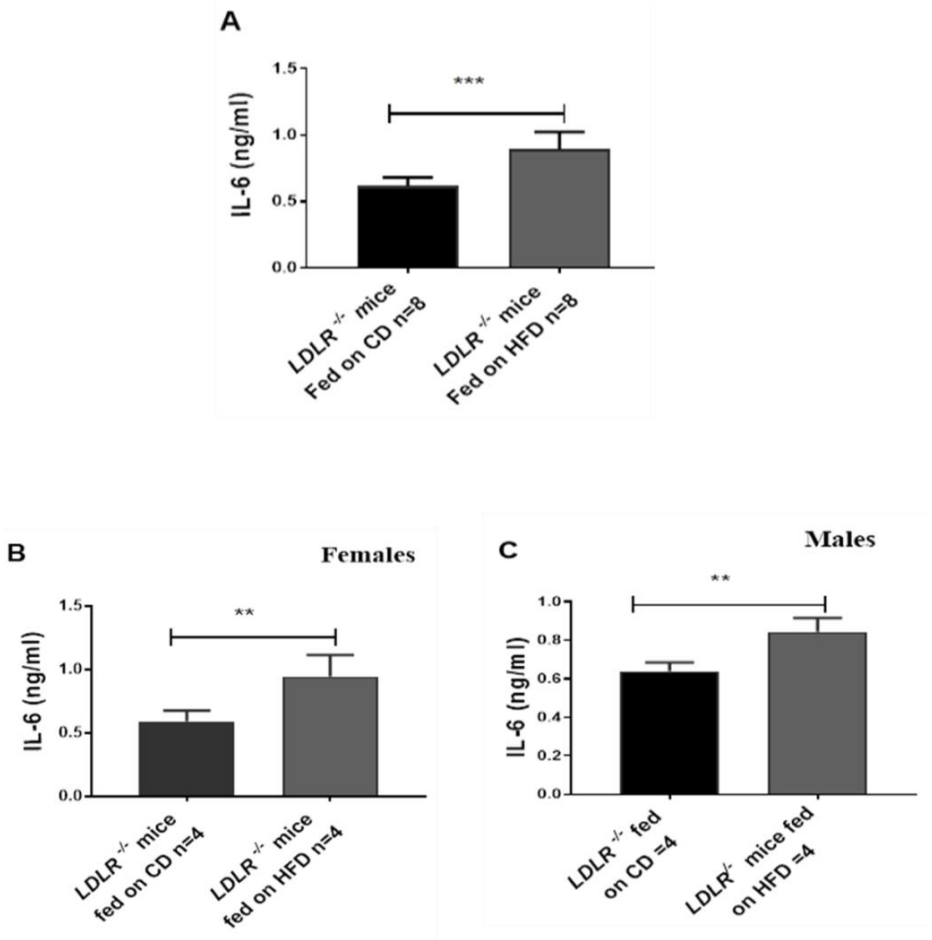


Figure 4-23: Effect of HFD feeding on IL- 6 protein in sera of *LDLR*$^{-/-}$ tumour bearing mice.

ELISA was used to detect the concentration of IL-6 after consuming dietary rich in fat for 12-weeks in *LDLR*$^{-/-}$ tumour bearing mice (**A**). (**B** and **C**) the measurements of this cytokine in sera of females and males groups fed on fatty diet or control diet, respectively. Results are presented as means ±SEM from triplicate determinations n = (3), and statistical analysis was performed by unpaired t test ($^{*}p < 0.05$).

4.4.2.4.2 Fatty diet elevates TNF-α level in sera of *LDLR*$^{-/-}$ tumour bearing mice

The effect of a 12-week HFD on TNF-α concentration in the sera of *LDLR*$^{-/-}$ tumour bearing mice was examined. There was a significant increase in TNF-α production in mice fed a HFD compared with those fed a CD (p=0.0001) (**Figure 4-24A**). Similarly, TNF-α was significantly increase in the HFDs of female (p= 0.0009) and male (p= 0.0028) mice fed compared with CD fed mice (**Figure 4-24B and C**).

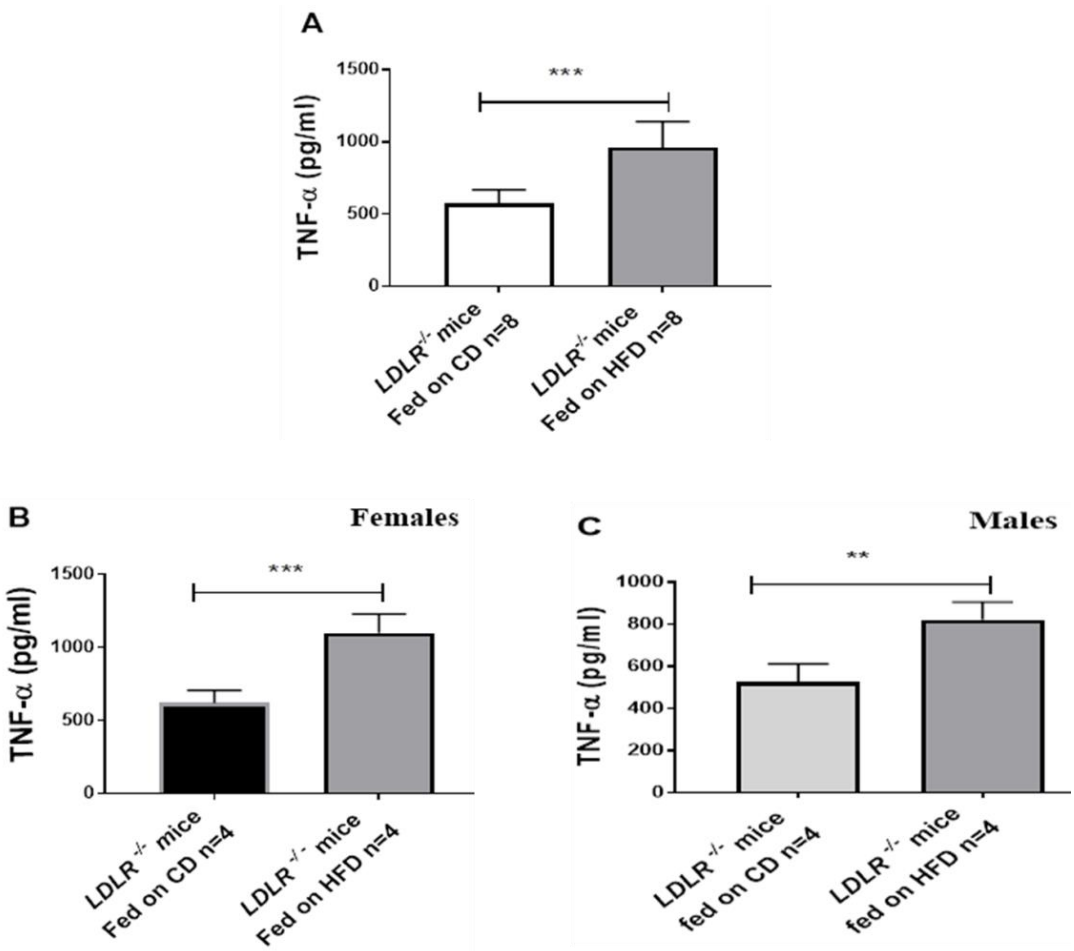


Figure 4-24: Impact of dietary fat on TNF-α protein production in *LDLR*$^{-/-}$ tumour bearing mice.

(**A**) TNF-***α*** measurements in murine serum of *LDLR*$^{-/-}$ tumour bearing mice fed on a HFD for 12-weeks and compared with CD feeding mice using ELISA. The levels of TNF-α in males and females group, respectively (**B** and **C**). Results are presented as means ±SEM from triplicate determinations n = (3), and statistical analysis was performed by unpaired *t* test ($^{*}p < 0.05$).

4.4.2.4.3 Diet rich in fat up-regulates circulating levels of adipokine Leptin

To investigate the effect of a 12-week HFD on the levels of Leptin in *LDLR*$^{-/-}$ tumour bearing mice ELISA assay was done. There was a significant increase in the circulatory levels of Leptin in tumour bearing mice fed on a HFD compared to those fed on a CD (*P*=<0.0001) (**Figure 4-25A**). Furthermore, both male and female groups had a significant increase in Leptin levels when fed a HFD compared with a CD (*p*= 0.0010) (**Figure 4-25B and C**).

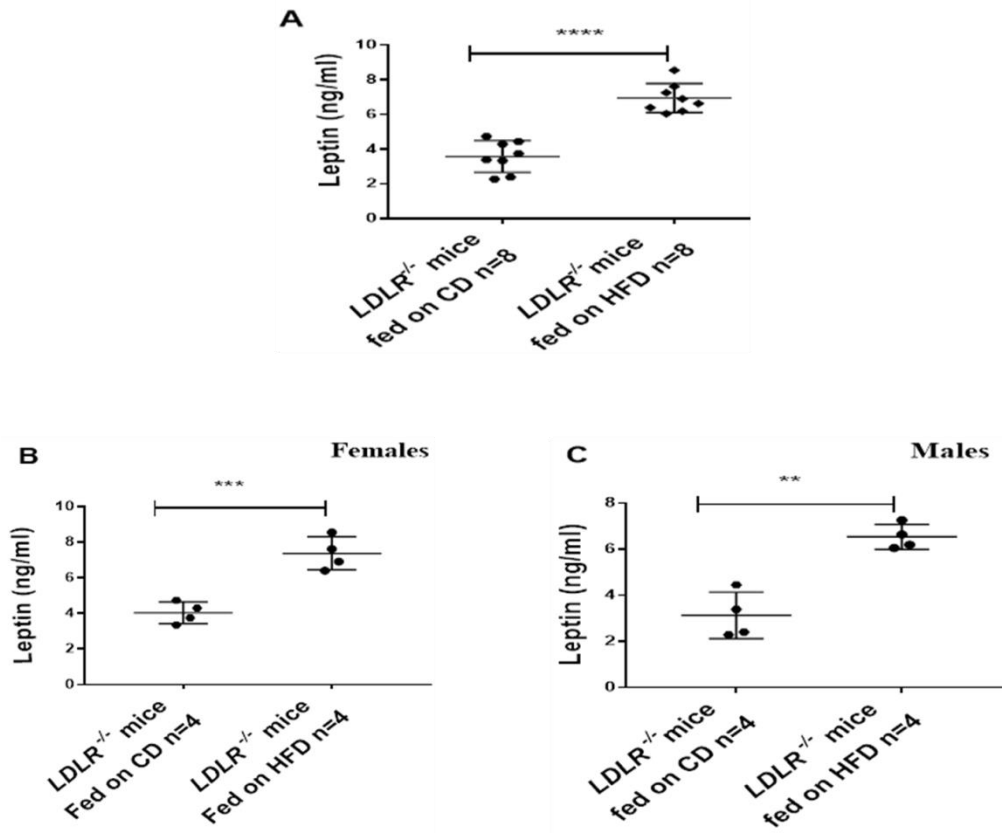


Figure 4-25: Quantitative measurements of adipokine Leptin concentration in sera of *LDLR*$^{-/-}$ tumour bearing mice fed on HFD or CD.

(**A**) The effect of HFD on Leptin levels in tumour bearing mice, while, (**B** and **C**) Levels of this adipokine in females and males tumour bearing mice, respectively. The data are represented means ±SEM from triplicate measurement n= (3). Statistical analysis was performed by unpaired *t* test. ($^{*}p < 0.05$).

4.4.2.4.4 HFD down-regulates the expression of Leptin receptors (ObRs)

Western blot analysis was used to detect the expression of Leptin receptor isoforms in tumour lysates of *LDLR*$^{-/-}$ mice fed on HFD or CD. A pool of 3 tumour lysates from each *LDLR*$^{-/-}$ mice fed on HFD diet (58R3) and control diet (58R1) were analysed by Western blotting with indicated antibodies. 20 μg of each lysate were subjected in 10 % SDS PAGE, β-actin was used as loading control. Expression of all Leptin receptor isoforms (ObRs) was detected in all tumour lysates analysed. However, expression of ObRs isoforms was significantly reduced in the HFD group compared to a CD (**Figure 4-26A and B**).

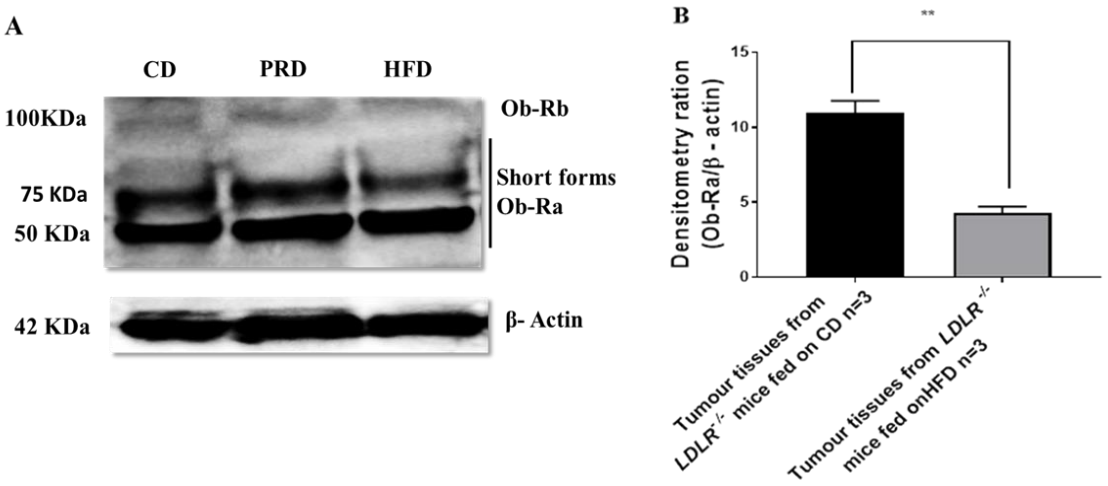


Figure 4-26: Western blot analysis of Leptin receptor isoforms (Ob-Rs) in melanoma tumour tissues of *LDLR*$^{-/-}$ mice fed on different diets

(**A**) Blot shows expression of Ob-Rs isoforms in tumour tissues of *LDLR*$^{-/-}$ mice. (**B**) Densitometry analysis of Ob-Ra protein expression. The data are represented as means ±SEM (n=2). The results are presented as a ratio of densitometry analysis of Ob-Ra to β-actin using Image J software and the data analysis was performed by unpaired *t* test. ($^{*}P < 0.05$).

4.4.2.4.5 High Fat Diet increases CCL2 (MCP-1) levels in *LDLR*$^{-/-}$ tumour bearing mice sera

The aim of this experiment was to investigate the effect of chronic consumption dietary fat on the secretion of CCL2 in *LDLR*$^{-/-}$ tumour bearing mice. There was a significant increase in CCL2 in the sera of tumour bearing mice fed a HFD compared to those fed a CD (**Figure 4-27A**). The results also showed that significant increase in the secretion of CCL2 in females group fed on HFD compared to their counterpart fed on CD **(Figure 4-27B)**. Similarly, the males group fed a HFD had significantly higher levels of CCL2 in contrast to those fed on CD **(Figure 4-27C)**. These results are consistent with previous results which showed an increase in tumour size of mice fed on HFD (**Figure 4-19**).

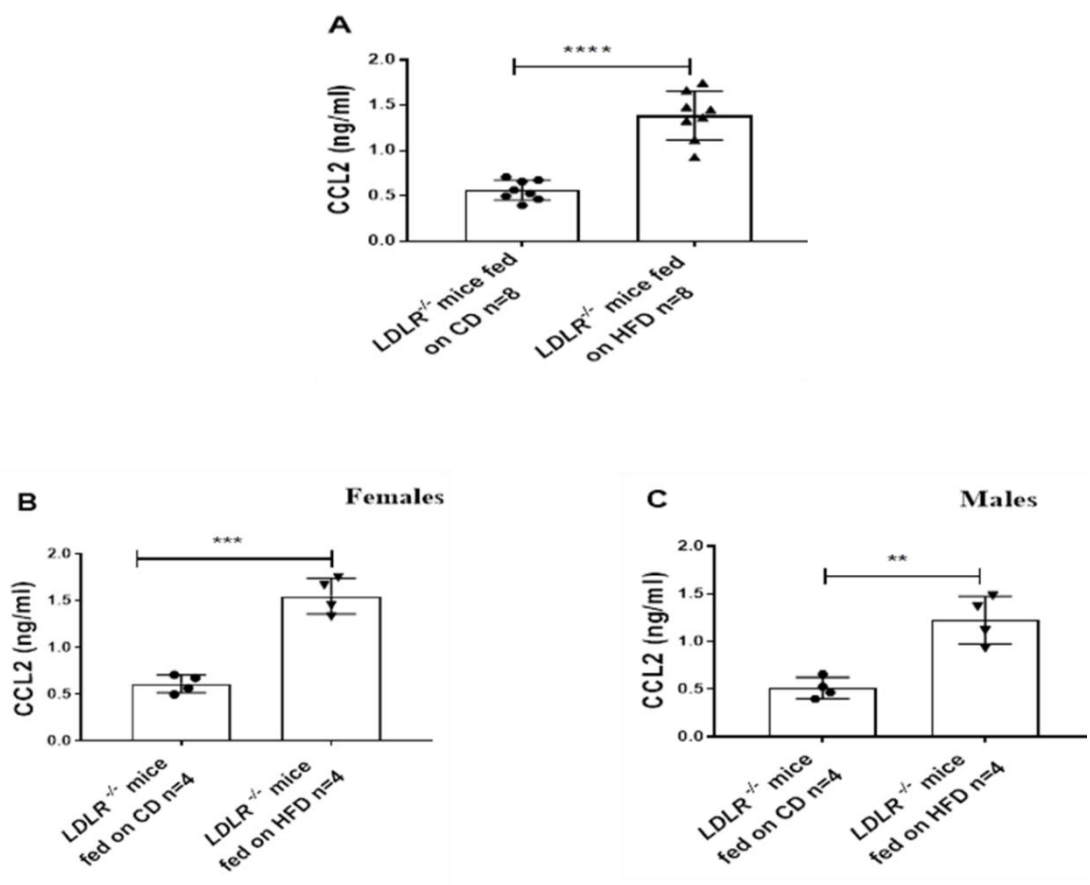


Figure 4-27: Quantitative measurements of CCL2 in sera of *LDLR*$^{-/-}$ tumour bearing mice fed on HFD or CD.

(**A**) Serum concentrations of CCL2 in both groups detect by sandwich ELISA. (**B** and **C**) the levels of this chemokine in females and males fed on fatty diet or control diet, respectively. Data are presented as means ±SEM from triplicate determinations n= (3). Statistical analysis was performed by unpaired *t* test ($^{*}p < 0.05$).

4.4.2.4.6 Chronic consumption dietary rich in fat down-regulates GPR120 mRNA expression in *LDLR*$^{-/-}$ tumour bearing mice

The impact of consuming a HFD on GPR120 mRNA expression levels in melanoma tumour tissues of *LDLR*$^{-/-}$ mice was examined. There was a significant reduction (p= 0.0286) of expression of GPR120 mRNA in the tumour tissues of obese mice on a HFD compared to a CD fed mice (**Figure 4-28**).

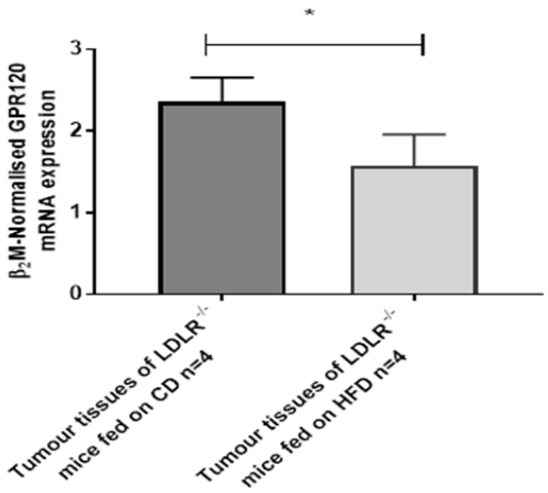


Figure 4-28: Quantitative calculation of mRNA expression of GPR120 receptor in the tumour tissues of *LDLR*$^{-/-}$ mice fed on HFD or CD.

The gene expression was normalised to β2M as reference gene expression, data are presented as means ±SEM from duplicate determinations (n= 2), and the data analysis was performed by unpaired *t* test ($^{*}P < 0.05$).

4.5 Discussion and conclusion

Several studies have shown strong positive correlations between diet-induced obesity and the incidence and development of several types of cancers such as breast, colon, pancreatic and cervical and melanomas (Calle *et al.*, 2003; Calle and Kaaks, 2004). Despite the well-established association between obesity and cancer, the triggering factor (or factors) linking these two conditions have yet to be identified. This thesis explores the inflammatory microenvironment as a potential mechanism linking obesity with enhanced tumour growth. Several pieces of evidence indicate the key role of lipids as a mediator of excessive fat accumulation and chronic inflammation (Teixeira *et al.*, 2016).

Although the effects of adipocytes on melanoma cell growth have been studied (Jung *et al.*, 2015; Kwan *et al.*, 2014; Kushiro *et al.*, 2012), the impact of obesity on malignant melanoma growth and the architecture of the tumour microenvironment in hyperlipidemic mice has yet to be investigated. In this study we used Low-density lipoprotein (LDL) receptor knockout mice (*LDLR*$^{-/-}$) as a genetic model of familial hyperlipidaemia. (Kennedy *et al.*, 2010). Tumours from hypercholesteraemic mice displayed reduced apoptosis and had increased microvessel density, linking hypercholesterolemia to the induction of angiogenesis and an increase in tumour growth *in vivo* (Pelton *et al.*, 2014). Therefore, we postulated that Low Density Lipoprotein receptor deficient *LDLR*$^{-/-}$ mice fed a HFD would be prone to larger tumours compared to those fed on a control diet. Growing evidence suggested that there are sex differences in obesity-induced complications such as diabetes, cardiovascular disease and metabolic syndrome in human and experimental animals.

Furthermore, the regulation at the genetic/molecular level and sex hormones such as estrogen played important role in the variation of cancer occurrence between sexes (Kim *et al.*, 2018). Additionally, these differences also influenced susceptibility to obtain weight gain between genders (Shi and Clegg, 2009). Based on the aforementioned findings, this study has considered these differences between genders and assessed the effect of HFD on melanoma tumour growth in both sexes. We analysed the effect of feeding mice for 4-and 12-weeks a HFD (20% protein, 36% fat, and 35% carbohydrate) on melanoma tumour growth and tumour microenvironment in female and male *LDLR*$^{-/-}$ mice. After 4-weeks consumption of a HFD, the results indicate a

significant increase in weight gain and increase in abdominal fat in males groups compared to their males fed a control diet. However, the female mice did not reach statistical significance within this timeframe. Therefore, the more rapid weight gain in the male mice may explain why the tumours were larger and more T-regs were present in that timeframe. The results also showed that increasing the HFD to 12-weeks induced increased body weight gain in both genders compared to control diet-feeding mice.

In addition, macroscopic observation showed higher accumulation in subcutaneous fat, mesenteric fat, and retroperitoneal fat. Interestingly, females appear to gain more weight after chronic consumption dietary fat compared to males. In agreement with our findings, Medrikova *et al.* (2012), showed that female C57BL/6N mice accumulated more fat and showed larger adipocytes compared with males after long-term (35-weeks) HFD feeding . Our results also concur with Chakraborty *et al.* (2016), found that long term HFD (12,25 and 27-weeks) induced significant increases in weight gain, hyperleptinemia, hyperinsulinemia, and estrogen levels along with increases in size of adipocytes in (C57BL/6J) female mice(age 14-16 weeks). Fitzgerald *et al* (2018), reported that females have the ability to store fat and have higher amounts of subcutaneous fat than age-matched males. Furthermore, a previous laboratory –based work (PhD thesis Kheder , 2017), found that dietary fat (36% fat) for 5-weeks and 10-weeks significantly increase the body weight gain in *LDLR*$^{-/-}$ mice (C57BL/6J) (3 months of age). Consistent with these observations, Jung *et al*. (2015), demonstrated significantly increased body weight gain after 16-weeks feeding HFD compared to those fed on a CD in C57BL/6N mice. However, our findings are partially inconsistent with Pettersson *et al.* (2012), who found no significant differences in weight gain between sexes after 14-weeks feeding HFD but their weight was significantly more than mice fed a control diet.

Noticeably, no difference was seen in mice weight after syngeneic injection with cancer cells (B16-F10). The results also showed that circulating non-esterified fatty acids (NEFA) levels were significantly higher in mice fed on a HFD for 4-weeks compared to CD and these levels were higher in males compared with females. This result agrees with a very recent report showing that mice given a HFD for five weeks developed fatty liver disease, with increased insulin and NEFA levels and these effects were higher in *LDLR*$^{-/-}$ males compared to *LDLR*$^{-/-}$ females (Kheder *et al.*, 2017). Analysis of tumour weight and tumour size after 4-weeks consumption of dietary fat revealed that male

mice have significantly bigger tumours compared to those fed on a control diet. Furthermore, in comparison with CD females, HFD females have larger and heavier tumours but not significant. Afterwards, we sought to investigate the effect of chronic consumption of dietary fat on tumour growth.

The result revealed that a chronic HFD induced a greater increase in tumour weight and size in both genders in comparison with those fed on a control diet. Additionally, females had larger tumour burdens than males fed the same diet. These findings were supported by Jung *et al* (2015), who found that long term consumption of dietary fat (60% of kcal from fat for 16 weeks) significantly increased cell proliferation, decreased apoptosis in subcutaneously injected melanoma B16-F10 cells and thereby enhanced solid tumour growth. Similarly, Pandey and collaborator (2012), who demonstrated that a 6-month HFD increased caveolin1 and fatty acid synthase (FASN) expression which was associated with larger tumours in C57BL/6J mice (4–5 weeks of age) compared to lean mice.

4.5.1 The impact of a HFD on cytokines expression

The effect of a 4-week HFD on cytokine secretion was investigated in the sera of *LDLR*$^{-/-}$ tumour bearing mice. Proteome profile results showed increases in the production of the chemokines CCL11, CCL2/MCP-1, CCL21/6Ckine, CCL22/MDC, CCL5, and Chemerin /CCL12 in the sera of mice fed on a HFD compared to those fed a CD. Several publications identified the important role of chemokine in tumour growth and progression in various types of cancers and melanoma specifically. Melanoma cells express CCL5/RANTES and it is up-regulated by TNF-α (Mrowietz *et al.*, 1999). Murine experiments showed that melanoma cells that expressed CCL5 formed highly aggressive tumours in nude mice in a concentration- dependent manner with expression favouring tumour progression (Payne and Cornelius, 2002). Disruption of CCR5/CCL5 signalling in mouse models impairs intra-tumoral T-regs accumulation and slows tumour development (Tan *et al.*, 2009).

Similarly, in human colon cancer, the levels of CCL5 are positively correlated with T-reg frequencies and impaired CD8T cell responses (Chang *et al.*, 2012). Furthermore, Wiley *et al.* (2001), who found that chemokine ligand-receptor CCL21/CCR7 facilitated murine B16 melanoma cell migration into the sentinel lymph nodes. Interestingly,

Shields *et al.* (2010), who found that B16 melanomas engineered to express higher levels of CCL21 recruited high numbers of T-regs and progressed rapidly compared to tumours expressing normal or lower CCL21 levels. Expression of CCL11 has been also shown to mediate angiogenesis *in vivo* and induces endothelial cell migration *in vitro.* Shi and co-worker (2017), found that CCL12 is a major cytokine that recruits monocytic cells (mo-MDSCs) to the pre-metastatic niche in lungs of tumour-bearing mice. Additionally, expression of chemokines CCL22 (a ligand for CCR4) by tumour cells, plays an important role in the recruitment and enrichment of T-regs (Ondondo *et al*., 2013). Using a mouse model, blockade of the CCL22/ CCR4 axis leads to the reduction in T-reg frequencies and a concomitant increase in anti- tumour activity (Ishida *et al.*, 2006; Pere *et al.*, 2011).

Our results also showed an increase in the production of growth Factors and tumour angiogenesis factors (Angiopoietin1, Angiopioetin-2, Angiopioetin-like3, M-CSF, VEGF-A, FGF acidic, Gas 6/TAM, EGF, DKK-1). Tumour formation efficiency and tumour progression are enhanced by overexpression of DKK1, whereas knockdown of DKK1 significantly reduced both migratory and invasive abilities of Hepatocellular carcinoma (HCC) (Tao *et al.*, 2013; Tung *et al.*, 2011). Brunckhorst *et al.* (2014), found that Angiopoietin promotes accumulation of cancer-associated fibroblasts and tumour angiogenesis in the ovarian cancer microenvironment, as well as enhancing ovarian cancer cell proliferation and invasion *in vivo.* Moreover, consistent with our finding, it has been demonstrated that crosstalk between murine melanoma B16-F10 and TAM increases the secretion of M-CSF, CCL2, VEGF-A, and thereby stimulates tumour cell migration, angiogenesis and lymph angiogenesis in an obese melanoma mouse model (Jung *et al.*, 2015). Importantly, blocking of VEGF-A not only decreases angiogenesis but also induces a reduction in T-regs infiltration in mouse models resulting in enhanced vaccine-induced immune responses (Li *et al.*, 2006). The effect of Gas6/TAM on promoting cell survival, aggregation, migration and growth has been identified in many types of cancers such as ovarian carcinoma, lung cancer, gastric cancer, prostate cancer, renal cell carcinoma, breast cancer and melanoma (Wu *et al.*, 2017). Furthermore, the results showed an increase in the cytokines and pro-inflammatory markers such as IL-1alpha, IL-4, Fetuin A /AHSGm, CXCL5, C reactive protein, Reg3G, Osteopontin (OPN),RBP4, Osteoprotegerin, CD257/BAFF, Pentraxin-3 (PTX3). Experimental animals and clinical studies found up regulation of these cytokines under inflammation

conditions and they play an important role in tumour growth and progression in various types of cancers such as pancreatic, gastric, breast, colorectal, cervical and melanoma (Koizumi *et al.*, 2013; Liu *et al.*, 2017; Recchi and Seabra, 2012; Ying *et al.*, 2016). Dabrowska *et al.* (2015), who reported that high fetuin-A correlated with metabolic syndrome and could be a predictive marker for poor survival in hepatocellular cancer patient.

The proteomic analysis also showed that after 4-weeks feeding diet rich in fat, the secretion of leptin and Resistin was significantly higher. However, the production of adipokines Adiponectin was significantly low. Published study reported that circulating levels of leptin and Resistin was negatively correlated with Adiponectin in HFD-induced obesity (Dalamaga *et al.*, 2012). Furthermore, *In vivo* study showed that increases in Adiponectin levels are inversely correlated with risk of obesity-associated malignancies (Hefetz-Sela and Scherer, 2013; Körner *et al.*, 2007). The results also showed increase in Enzymes (CD26/DPPIV, Cystatin C, Chitinase 3-lik 1, Myeloperoxidase, MMp-2, MMp-3, MMp-9, Proprotein, and Convertase9). The expression of these was found to be positively correlated with increases in the body mass indices (BMI) and inflammation and pathogenesis of various cancers (Castillo-Tong *et al.*, 2014; Unal *et al.*, 2010; Huang, 2018; Aliyari Serej *et al.*, 2017).

4.5.2 The effect of HFD on adipokines level and their receptors

Alteration in the secretion of adipokines, cytokines secreted by the adipose tissue during chronic obesity has been demonstrated in many published studies (Malvi *et al.*, 2018; Freitas Lima *et al.*, 2015; Bluher, 2014). This alteration leads to an increase in the levels of pro-inflammatory adipokines and a simultaneous decrease in anti-inflammatory adipokines (Khasawneh *et al.*, 2009; Lumeng and Saltiel, 2011). Several adipokines such as TNF-α, IL-6, IL-8, and MCP-1 (CCL2) have been implicated in tumour growth and progression (Guerrero *et al.*, 2010).

Our ELISA data revealed significant increases in circulating IL-6, TNF-α, Leptin as well as the chemokine CCL2 (MCP-1) in sera of obese *LDLR*$^{-/-}$ tumour mice fed on a HFD for 12-weeks compared to lean mice. Transcription nuclear factor kappa B-dependent (NF-*κB)* has important roles in the progression of malignant phenotypes, and aberrant activation of NF-*κB* is found in various types of tumours (Romashkova and

Makarov, 1999; Song *et al.*, 2011). The function of NF-*κB* signalling in cancer development is mediated through tumour-associated angiogenesis and inflammation (Strieter, 2005). Importantly, the release of fatty acids from expanded adipose tissues can activate toll-like receptor (TLR-4) on the macrophage plasma membrane, leading to an increase in NF-*κB* expression of pro-inflammatory genes, including TNF-α, IL-6 and CCL2 (Iyengar *et al.*, 2016). Furthermore, preclinical data demonstrated that intake of dietary fat high in saturated fat can alter the intestinal microbiota, leading to TLR-4 activation, inflammation, and insulin resistance (Caesar *et al.*, 2015).

Additionally, apart from activation of TLR-4, pyrin domain–containing 3 (NLRP3), inflammasome in macrophages can be activated by fatty acids and cholesterol crystals which mediate the release of pro-inflammatory proteins (Ralston *et al.*, 2017). Mature adipocytes undergo dedifferentiation or apoptosis after contact with tumour cells, which leads to activation of pro-inflammatory cytokine secretion by macrophages within the tumour microenvironment (Dirat *et al.*, 2011; Andarawewa *et al.*, 2005). Adipocytes dedifferentiation leads to interleukin (IL)-6 production, which promotes survival and proliferation of cancer cells and thereby supports tumour growth (Wang *et al.*, 2017a). In a genetic mouse model of pancreatic cancer HFD induced obesity and secretion of TNF-α and IL-6 showed a higher rate of β-oxidation and early tumour growth and inhibiting TNF signalling could reduce these effects (Khasawneh *et al.*, 2009). My data is supported by previous observations showing that a diet rich in fat increases melanoma cell growth in the bone marrow by inducing osteopontin and interleukin 6 (Chen *et al.*, 2016). Similarly, Dirat *et al.* (2011), found IL-6 secreted from cancer-associated adipocytes and promoted tumour progression in breast cancer models. Furthermore, consuming dietary fat for 4 weeks led to increased adipose tissue expansion and resulted in the elevation of plasma CCL2 (MCP-1) which continued to rise during 20 weeks of a HFD (Chen *et al.*, 2005). Circulating levels of MCP-1 are generally increased in obese patients compared to lean controls (Catalan *et al.*, 2013). Within the tumour microenvironment, elevated CCL2 is associated with increased TAMs infiltration. In breast cancer mouse model, obesity enhances tumour growth by CCL2-mediated macrophage recruitment and Angiogenesis (Arendt *et al.*, 2013a).

The ability of CCL2 to attract tumour-promoting and immunosuppressive cell types such as TAMs and MDSC to tumour microenvironment (TEM), provides a potent rationale for attempting to therapeutically reduce CCL2 levels in the setting of

established neoplasms (Li *et al.*, 2013). Furthermore, in tumour tissues of HFD-fed mice, increased adipocytes enhance tumour cells to secrete CCL2 which increases the recruitment of M2 phenotype macrophages and thereby promotes melanoma tumour growth (Jung *et al*. 2015). According to these findings, I interpret that obese mice bearing tumour have significantly higher levels of chemo-attractant CCL2 compared to lean tumour bearing mice due to increase of infiltrating immune cells.

Leptin is one such adipokine which is increased significantly in obese subjects and is known for its ability to regulate energy expenditure (Stone *et al.*, 2018). Previously, our proteome array showed significant increase in Leptin levels after a 4-week HFD. Therefore, we addressed whether a longer term HFD would significantly increase the levels of circulating Leptin and how it might affect the expression of its receptor. ELISA results showed a significant increase in Leptin levels in *LDLR*$^{-/-}$ mice after a 12-week HFD. Furthermore, obese female mice had higher circulating Leptin levels compared to obese males and this may be due to an increase in body weight of females. Leptin concentrations are largely correlated with BMI in humans and rodents (Greenberg and Obin, 2006). Munzberg *et al.* (2004), demonstrated increase in Leptin concentration in obese mice fed on a HFD. In case-control study, Gogas *et al.* (2008), reported a positive correlation between circulating levels of Leptin and increased risk of melanoma. Amjadi and collaborators (2011), observed that Leptin enhanced vasculogenesis via increase nitric oxide (NO) production and circulating endothelial progenitor cells (EPC) which promotes tumour growth in melanoma mouse models. However, Brandon *et al*. (2009) found that obesity markedly increases melanoma tumour growth in obese melanocortin receptor 4 knockout (*MC4R*$^{-/-}$) and Leptin deficient (*ob*$^{-/-}$) mice independently of host Leptin and high leptin levels may accelerate tumour growth.

In vitro data showed less expression of ObRs in the B16-F10 cells treated with serum derived from mice fed on a HFD compered to serum form CD fed mice. Based on this data I investigated the effect of HFD on expression of Leptin receptors (ObRs) in *LDLR*$^{-/-}$ tumour bearing mice. Western blot results for tumour lysates prepared from the tumours of mice fed on CD and HFD showed expression of all Leptin isoforms (ObRs). To our knowledge this is the first study to show the expression of all Leptin isoforms in mouse B16 melanoma tumours. The results also showed significantly less abundance of Leptin receptor (ObRa) in obese mice compared to lean mice. Published study reported

that long (ObRb) and short (ObRa) Leptin receptor isoforms play essential roles in mediating leptin signalling and the transport and degradation of Leptin respectively (Uotani *et al.,* 1999). An *in vivo* study of pancreatic cancer showed that leptin promotes pancreatic tumour growth and migration through the activation of a short isoform ObRa mediated by PI3K/AKT pathway signalling (Mendonsa *et al.*, 2015).

The comparison between the expression of fatty acid receptors (GPR120) in tumour tissues of obese and lean *LDLR*$^{-/-}$ mice was done using qPCR. There was a significant reduction in GPR120 mRNA expression in tumour tissues of obese mice compared to lean mice. Recently, G-protein-coupled receptor 120 (GPR120) has received a great attention due its potential role in the regulation of metabolic and inflammatory diseases such as obesity and type 2 diabetes (Wu *et al.*, 2013). Accumulating evidence identifies a role for FFAR4 (GPR120) in tumourogenesis, migration, and metastasis in various tumour types (Houthuijzen, 2016). Li *et al*. (2015), who showed that FFAR1 (GPR40) and FAR4 (GPR120) activation induced cell proliferation of prostate cancer. The activation of GPR120 by FFAs protected tumour cells from DNA damaging chemotherapeutics *in vivo* (Houthuijzen, 2016). Wu *et al.* (2013), who found that GPR120 signalling acts as a tumour promoting receptor that induces angiogenesis and migration in human colorectal carcinoma. The mRNA analysis in this work points to a reaction of transcription levels to obesity and implies an involvement of the GPR120 receptor.

4.5.3 Flow cytometry analysis for Treg populations in obese *LDLR*$^{-/-}$ melanoma mouse model

There has been an explosion of literature focusing on the role of regulatory T cells (T-reg) in shaping the immune response to tumours (Ha, 2009). Recently, the most reliable marker to identify T-regs is the forkhead box transcription factor (Foxp3). This marker expressed specifically in murine $CD4^+$ T-regs and it is essential to their lineage identity and function (Klages *et al.*, 2010). Therefore, the present study used $CD4^+CD25^+$-$FOXP3^+$ markers to identify T-regs. T-regs express the transcription factor Foxp3 were basically identified for their ability to prevent organ- specific autoimmune disease in mice by suppressing self-reactive cells. Emerging evidence has found that T-regs play a crucial role in tumour immunology and may promote tumour, thereby having a

significant impact on the outcome of cancer patients (Dougan and Dranoff, 2009; Zou, 2006; Shen *et al.*, 2009).

FC analysis of regulatory T cells (T-regs) in *LDLR*$^{-/-}$ tumour bearing mice fed either a HFD or control diet for 4-weeks or 12-weeks was done, to understand the modulating effect of a HFD on tumour growth. Analysis of splenocytes in mice fed a HFD for 4-weeks showed a significant increase in T-regs in obese males group compared to the CD group. Although the proportion of T-regs in obese females was higher than CD counterparts, this did not reach statistical significance. Furthermore, the results also showed that chronic consumption of dietary fat for 12-weeks increased the frequency of splenic T-regs in all animals mirroring the increase in tumour weight and size. However, the frequency of these cells was higher in obese females who had larger tumours compared to obese male mice. Additionally, the data showed significantly increase in T-regs accumulation in spleen of mice bearing tumour compared to tumour free mice. Several publications found that percentage of T-regs dramatically elevated in tumours, blood and lymphoid organs of tumour bearing hosts (Liu *et al.*, 2009). Moreover, it has been found that *naïve* (tumour free) mice consuming a HFD showed a decrease in hepatic T-regs. However, the T-regs percentage in the spleen were not affected by this type of diet (Issazadeh-Navikas *et al.*, 2012). Data from experimental animals demonstrated an increase in T-regs in tumour bearing mice and their depletion or inhibition enhanced antitumor immunity (Ha, 2009). Our findings are also supported by a previous study which found that the proportion of $CD4^{+}CD25^{+}/CD4^{+}$ (T-regs) in spleen-derived lymphocytes was significantly increased in tumour bearing mice compared with tumour-free mice and this proportion increased with increasing tumour size (Liu *et al.*, 2005). Liyanage *et al.* (2006), found that the prevalence of $CD4^{+}CD25^{+}$/T-regs in the spleen is increased with tumour growth in a pancreas adenocarcinoma mouse model.

4.6 In summary

The results demonstrated that consumption of a diet rich in fat induced significant increases in weight gain in our mouse melanoma model and obese mice had larger tumours compared to lean animals. Additionally, HFD-induced obesity significantly altered chemokines and adipokines secretion in mice sera. However, it seems likely that a HFD has an indirect effect on splenic T-regs and the changes in their frequency corresponded to changes in tumour size. Thus, a combination of metabolic changes and an altered mediator profile leads to a TME that is more favourable to cancer growth and progression.

Chapter 5

In vitro effect of Vitamin D3 on tumorigenic phenotypes of co-cultured melanoma cells

In vitro effect of Vitamin D3 on tumorigenic phenotypes of co-cultured melanoma cells

5.1 Introduction

Vitamin D3 is a hormone produce by skin after exposure to sunlight before metabolic activation in liver and kidney to the active form of Vitamin D3 (1-alpha,25-dihydroxyvitamin D3) (Burns *et al.*, 2015). This Vitamin has been found to have immune modulatory actions and its role in the autoimmune disease, infections, cardiovascular disease and cancer was implicated in several epidemiological studies (Field and Newton-Bishop, 2011; Wasiewicz *et al.*, 2015). The function of this vitamin is mediated by Vitamin D3 Receptor (VDR), which are expressed not only by benign but also by malignant cells (Diesing *et al.*, 2006). Negative correlation was found between VDR expression and cancer progression: relatively non – malignant tissues showed high expression of VDR while, hyperplasia tissues showed reduction or complete loss of VDR expression (Trivedi *et al.*, 2017), showing a malignancy dependent response to circulating Vitamin D3 levels. It is now well-established that 1α, 2 5(OH) 2D3 exhibits anti-proliferative, pro-differentiating, anti-inflammatory, and pro-apoptotic activities in a tissue- and cell-specific manner (Lenders *et al.*, 2009; Bikle, 2010; Chiang and Chen, 2009). However, the association between VitD3 and melanoma has proven to be complex.

Due to its role in modulating the immune system, VitD3 has been potentially linked to obesity, which is a state of inflammation involving the secretion of pro-inflammatory cytokines from adipose tissues (Mehmood and Papandreou, 2016). Obesity may be associated with hypovitaminosis D3 (Nicolaides *et al.*, 2008). However, no clinical evidence supports this notion. Vitamin D3 being fat soluble, may sequester away from the circulation where it is measurable, in the obese. It has been suggested that VitD3 supplementation may lower the incidence of obesity but does not induce weight loss in obese individuals (Vanlint, 2013a). Profound progress has been made in understanding the complex action of VitD3 in transformed malignant cells; however, the influence of calcitriol on the nutrient utilisation of tumour is not fully understood (Abu el Maaty and Wölfl, 2017). No previous studies have investigated the effect of VitD3 on melanoma

tumours in the context of obesity. Therefore, the effect of Vitamin D3 (either supplemented diet or exogenously added in to HFD derived serum) was investigated on cell viability, cell migration, lipid deposition and the tumourgenic phenotype of a murine melanoma cell line (B16-F10).

5.2 Experimental plan

A series of *in vitro* experiments investigated the *in vitro* actions of VitD3 on the mouse skin cancer cell line B16-F10 (**Figure 5-1**). Concentrations of Vitamin D3 (1µM and 10 µM) were chosen based on those used by others in the literature (Wasiewicz *et al.*, 2015; Oliver *et al.*, 2012). Control cells were treated with the same concentration of Ethanol that is used in the preparation of VitD3.

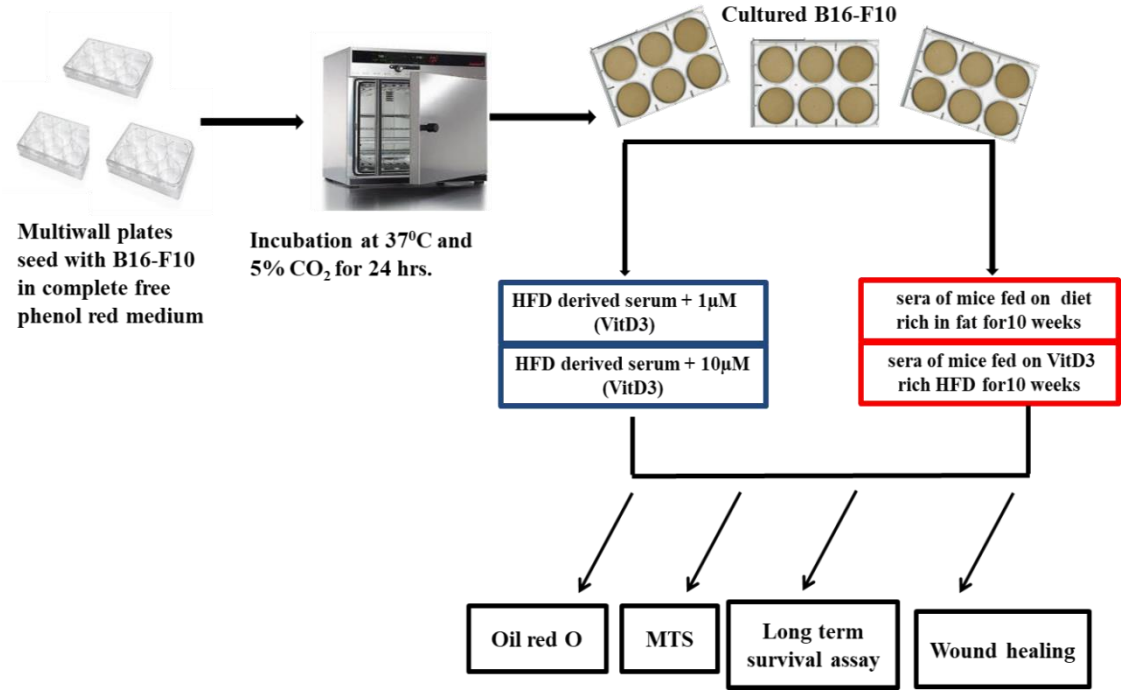


Figure 5-1: *In vitro* experimental strategy investigating the actions of vitamin D3 on murine melanoma cell line (B16-F10).

5.3 Results

5.3.1 Exogenous addition of VitD3 to HFD derived serum inhibites B16- F10 viability

Prior in *vitro* data showed that vitamin D3 has anti-proliferative effects on melanoma cells (Field and Newton-Bishop, 2011). Therefore, the effect of different doses of VitD3 was examined on B16-F10 proliferation in the presence of 2% HFD serum using an MTS assay. There was a significant reduction in the mitochondrial activity of cells treated with 1µM of exogenous VitD3 for 24 hours compared to control (p= 0.0279) (**Figure 5-2A and B**). However, the reduction in cell activity was significantly greatest at 10 µM (P= 0.0083) (**Figure 5-2A and B**), which indicated that a reduction in mitochondrial cell activity of B16-F10 was dose dependent.

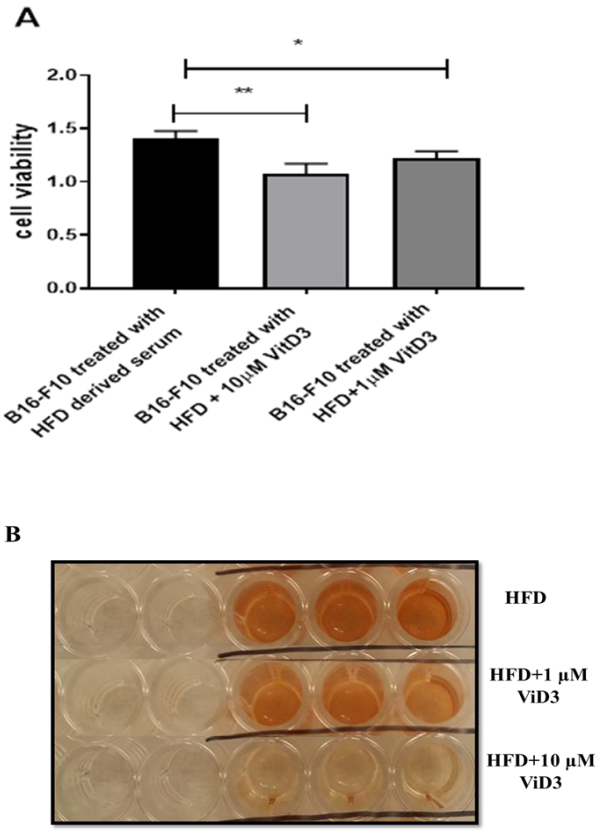
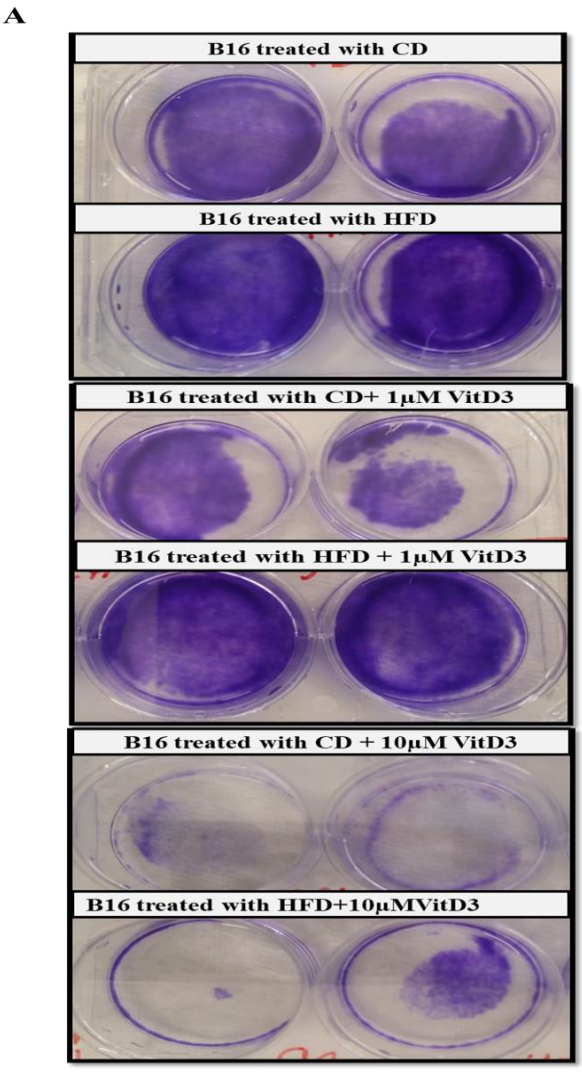


Figure 5-2: Mitochondrial cell activity of B16-F10 mouse melanoma cell line treated with vitaminD3.

Quantitative measurements of B16-F10 cell viability treated with Vitamin D3 (1and 10µM) were determined using an MTS assay. Change in colour occurred according to mitochondrial activity. The data are represented as the mean ± SEM of three independent experiments (n=3) and each sample was tested in triplicate. Statistical analysis was performed by one-way ANOVA/multiple comparisons test. ($^{*}p < 0.05$).

5.3.2 Long term exposure of exogenous VitaminD3 affectes melanoma cell adhesion

The effect of long-term exposure of Vitamin D3 at different doses was tested on the proliferation of B16 melanoma cells. The results showed a significant decrease in cell survival after treatment with 1µM VitD3 exogenously added to 2% CD serum compared to those treated with 1 µM VitD3 and 2% HFD sera (**Figure 5-3B**). However, the cell survival was higher in cells cultured in 2% sera from mice fed a HFD compared to those cultured with sera from CD fed mice **(Figure 5-3B)**. Interestingly, significant higher reduction in the growth of B16-F10 cells was noticed upon culturing them in 10µM VitD3 and CD sera compared to those cultured with CD only (**Figure 5-3B**). Crystal violet staining was used to verify long term survival of B16-F10 and the plates were photographed by Imaje J (**Figure 5-3A**).



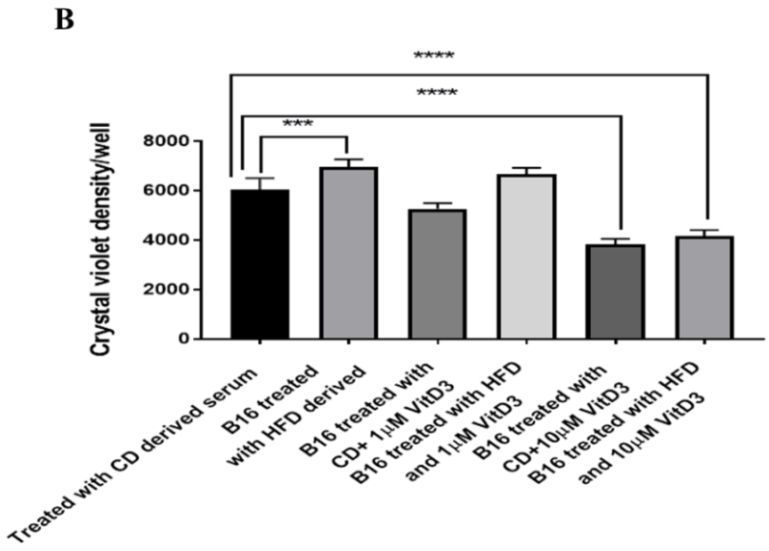


Figure 5-3: Crystal Violet analysis of B16-F10 cells under different treatments.
(**A**) Representative pictures of cultured B16 in serum collected from three C57BL/6J mice fed on CD and HFD, CD derived serum +VitD3 (1µM and 10 µM) and HFD derived serum +VitD3 (1µM and 10 µM). (**B**) Quantitative measurements of Crystal violet staining which represented the number of survival cell. The data are represented as the mean ± SEM of three independent experiments (n=3) and each sample was tested in duplicate. Statistical analysis was performed by one-way ANOVA/multiple comparisons test. ($^{*}p < 0.05$).

5.3.3 Exogenous addition of a HFD derived sera with Vitamin D3 decreases lipid accumulation in B16 melanoma cells *in vitro*

To investigate the effect of VitD3 on lipid inclusion of melanoma cancer cells (B16-F10) cells were preconditioned with sera from mice fed HFD exogenously supplemented with 1 or 10 µM Vitamin D3 for 48 hours. Oil Red O staining was performed, and Lipid-bound oil red-O was then quantified. The results showed that B16 cells cultured on HFD sera have significantly increased lipid accumulation than B16 control cells (p=0.0060). Additionally, exogenous addition of 10 µM VitD3 supplemented HFD significantly reduced lipid accumulation (p=0.0183). Finally, no significant differences were seen between untreated cells and cells stimulated with 1µM Vitamin D3 **(Figure 5-4)**. This experiment shows that there is a dose dependent effect of VitD3 on the ability of B16-F10 to store neutral lipids, implying an effect of VitD3 on a metabolic pathway.

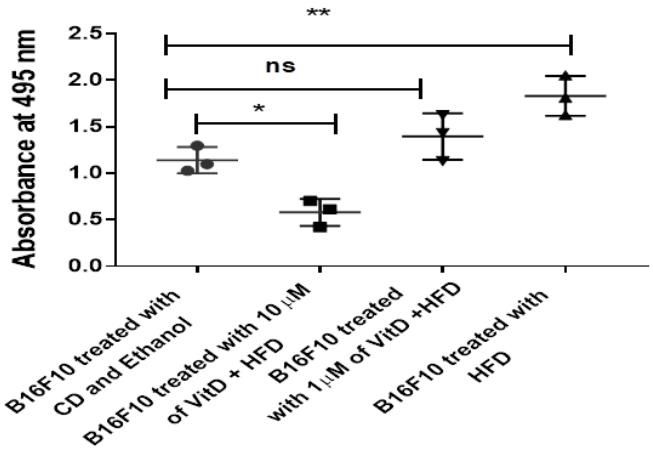


Figure 5-4: Quantitative measurements of lipid inclusion in B16-F10. Lipid content was measured by quantification of oil red-O staining.
Cells were stained with oil red-O and then washed in water and air dried. Lipid-bound oil red-O was then dissolved in isopropanol at 4^0C for 30 minutes, and then the absorbance of the oil red-O in isopropanol was determined at 495 nm. The data are represented as the mean ± SEM of three independent experiments (n=3) and each sample was tested in triplicate. Statistical analysis was performed by one-way ANOVA/multiple comparisons test. ($^*p < 0.05$).

5.3.4 Sera from mice fed on vitamin D3 supplemented HFD enhances the viability of B16 melanoma cells *in vitro*

Previous data showed that high dose of (10µM) when added to sera from mice on diet rich in fat significant inhibit cell viability. Therefore, the effect of vitamin D3 in mice sera fed on diet contained high fat and vitamin D3 for10-weeks on mitochondrial melanoma cells viabilities *in vitro* was investigated. B16-F10 cells were cultured in media containing 2% serum collected from experimental *LDLR*$^{-/-}$ C57BL/6J mice fed on a HFD or a HFD that contained VitD3. Interestingly, mitochondrial cell activity was increased significantly in cells cultured in serum from HFD +VitD3 feeding mice compared to those cultured in the serum from HFD fed mice (**Figure 5-5**). This is opposite to the findings that used serum with exogenous Vitamin D3 added for the *in vitro* assay prior to assessing cell viability.

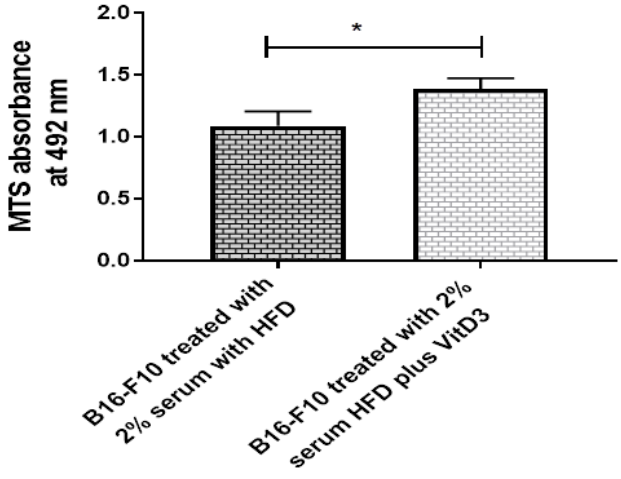
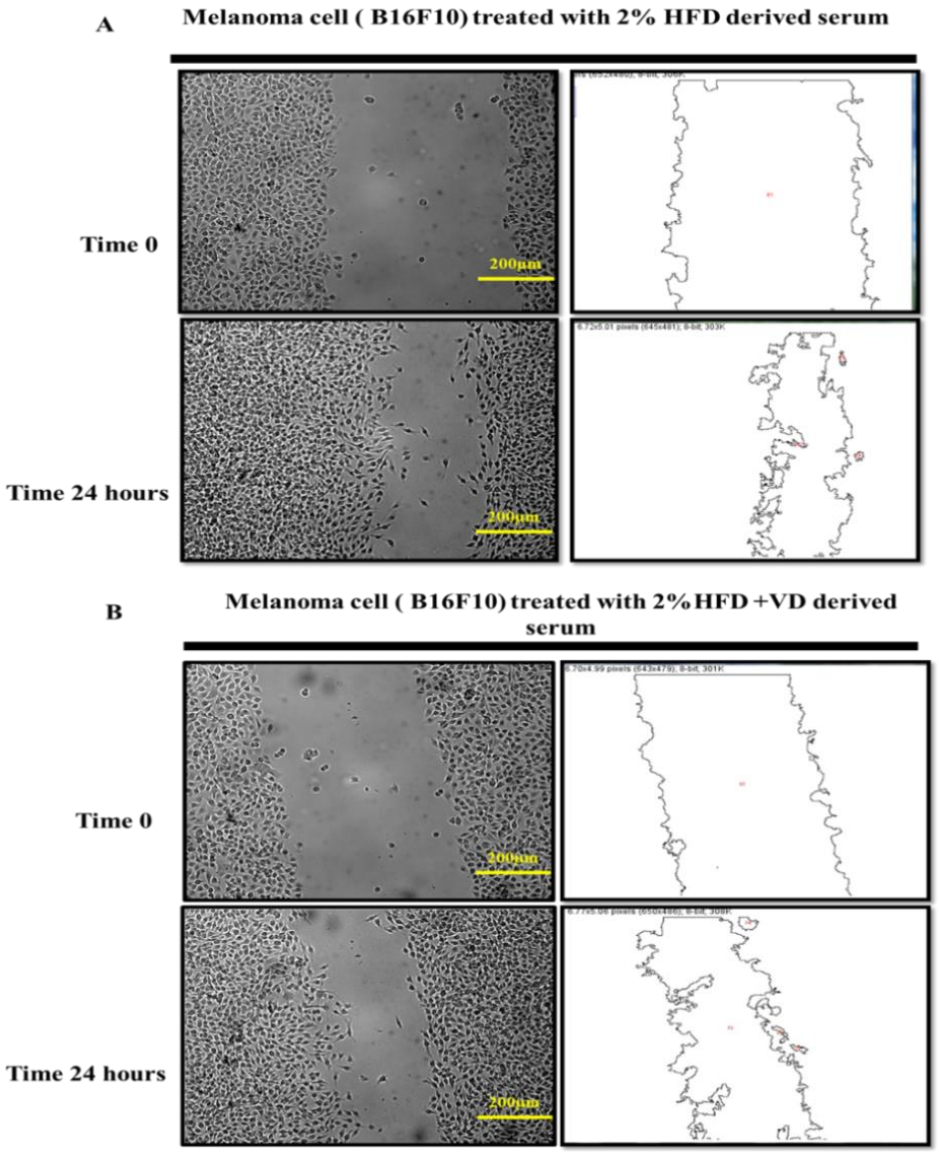


Figure 5-5: Mitochondrial cell activity of B16-F10 mouse melanoma cell line treated with 2% HFD and VitD3 or HFD alone derived serum.
MTS assay was used to quantify mitochondrial cell activity of B16-F10 and the data are represented as means ± SEM (n = 3). Statistical analysis was performed by unpaired *t* test. ($^{*}p < 0.05$).

5.3.5 Sera from mice fed a vitamin D3 supplemented HFD has no effect on the wound healing activity of B16 melanoma cells *in vitro*

Our data previously showed that HFD significantly enhanced cell migration of B16-F10 compared to those treated with CD after 24 hours. Therefore, to determine whether migration of B16-F10 cells would be modulated by VitD3 supplementation, B16-F10 cells were treated with 2% serum derived from mice fed on diet rich in fat supplemented with VitD3 or HFD only. After 24 hours of exposure, a phase contrast microscope was used to analyse the closing of the gap by these cells. The results showed that HFD serum slightly enhanced the migration ability of melanoma cells compared to those treated with VitD3 containing HFD derived serum at the time point of 24 hours (**Figure 5-6A and B**), as indicated by a~ 48% increase of coverage percentage in HFD group compared with the HFD plus VitD3 group (~42%) (**Figure 5-6C**).



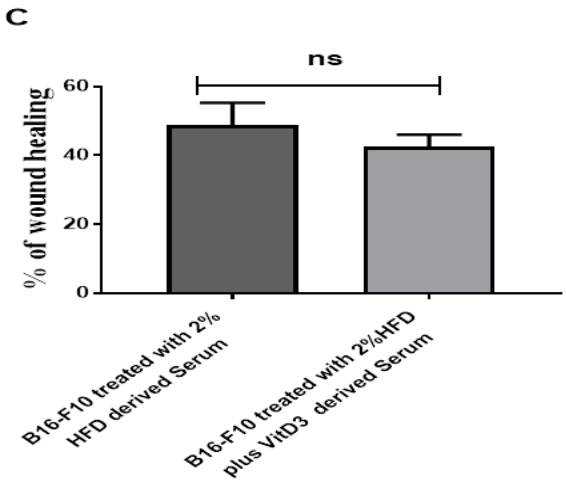


Figure 5-6: Effect of VitD3 on the migration ability of B16-F10.
(**A**) Images of wounded monolayer of B16-F10 treated with 2% serum derived from mice fed on HFD plus VitD3 diet taken at times 0 hours and 24 hours, while (**B**) Images of B16-F10 taken at times 0 hours and 24 hours after treatment with 2% HFD, respectively. (**C**) Percentage of wound healing was estimated by measurement of cell numbers within the wounded region after 24 hours of scratch. A representative image of at least 3 independent experiments (n=3) carried out in triplicate was shown. Statistical analysis was performed by unpaired *t* test ($^{*}p < 0.05$).

5.3.6 Exogenous addition of IL-10 and IL-6 cytokines in the sera of mice fed vitaminD3 rich HFD modulates B16 viability

Cytokines that are secreted in the inflammatory microenvironment are correlated with the pathogenesis of several cancers (Liu *et al.*, 2018). Furthermore, the role of VitD3 as an anti-inflammatory agent has been demonstrated (Krishnan and Feldman, 2011). Pervious data showed that B16-F10 cells treated with HFD derived serum and exogenous addition of recombinant IL-6 significantly enhanced cell viability compared to cells treated with control diet derived sera containing recombinant IL-6 (**Figure 3-12**). To investigate the effect of sera from mice fed a HFD containing VitD3 in the presence of added recombinant IL-6 or IL-10, proliferation assay of melanoma tumour cell (B16-F10) growth was done. There was a significant inhibition on mitochondrial activity of B16-F10 in the presence of IL-10 compared to those treated with serum derived from mice fed on HFD+VitD3 alone (p=0.0079) (**Figure 5-7**). However, cell viability showed significantly higher increase in the presence of IL-6 (p=0.0409) (**Figure 5-7**). So the effect of HFD serum can be further modulated by the presence of cytokines.

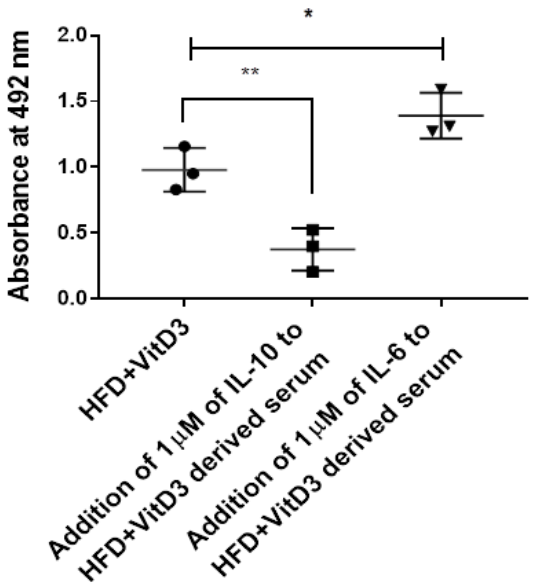


Figure 5-7: Impact of VitD3 derived serum in the presence of recombinant cytokines IL-6 or IL-10 on murine melanoma cells (B16-F10).
The data are represented as the mean ± SEM of three independent experiments (n=3) and each sample was tested in triplicate. Statistical analysis was performed by one-way ANOVA/multiple comparisons test. ($^{*}p < 0.05$).

5.4 Discussion

The sensitivity of murine B16-F10 melanoma to Vitamin D3 has been widely investigated with contradictory results. Published data reported that VitD3 induced anti-proliferative effect on some melanoma cell line *in vitro* (Field and Newton-Bishop, 2011). However, no previous experimental trial has investigated the effect of this hormone on melanoma in obese subjects. A colorimetric tetrazolium salt assay (MTS assay) was used to determine the effect of VitD3 on melanoma cell proliferation. The results showed a significant reduction in mitochondrial cell activity of B16-F10 at 1µM. However, this reduction was significantly greater at 10µM VitD3. These findings are consistent with Wasiewicz *et al.* (2015), who found that VitD3 induced significant inhibitory effect on B16-F10 cell growth at 1µM. Furthermore, a human breast cell line (MCF-7) showed inhibition in cell growth after treatment with >20 µM vitamin D3 (calcitriol) (Murray *et al.*, 2017). Treatment with sera derived from mice fed on VitD3 rich HFD diet induced a significant increase in cell proliferation compared to those treated with HFD derived sera, probably due to other soluble factors as I will discuss later.

In addition to enhanced proliferation, cell migration is a fundamental feature of cancer development, particularly during invasion and metastasis (Hughes *et al.*, 2008). The effects of treatment with a HFD containingVitD3 or a HFD alone on migration using wound healing assay was explored. The result revealed that there were no significant differences on B16-F10 cell migration between cells treated with HFD and VitD3 (~42% healing) and those treated with a HFD only (~48% healing). These data indicate that HFD-VitD3 had an effect on mitochondrial cell activity but not on cell migration. Supporting this data, Yudoh *et al.* (1999), who found that cell motility of B16-F10 was not influenced by Vitamin D3 after 48 hour incubation within a dose range of 0.01 to 100.0 nmol/L. Published study reported that an increase in lipid accumulation and *de novo* fatty acid synthesis are a common feature of aggressive cancer cells and targeting lipid metabolism could be considered a promising target for cancer prevention (Menendez and Lupu, 2007). Therefore, the ability of Vitamin D3 to reduce lipid accumulation was investigated and revealed a significant attenuation in lipid inclusion in B16-F10 cultured with VitD3 + HFD derived serum at 10 µM for 48 hours. In contrast, treatment with the 1µM showed an increase in lipid inclusion but did not reach the significant compared to control. While there was significant lipid inclusion was seen

in B16-F10 treated with HFD derived serum compared to other conditions. These findings concur with Wilmanski *et al.* (2017), who demonstrated that treatment with 10nM VitD3 inhibited neutral lipid accumulation in malignant breast epithelial cells at five and seven days in a time dependent manner. Furthermore, Chang and Kim (2016), found that VitD3 significantly reduced neutral lipid accumulation in 3T3-L1 adipocytes. In contrast to this thesis, Wang *et al*.(2013), described that prostate cancer cells incubated with 100nM VitD3 for six days had increased neutral lipid accumulation, as measured by Oil Red O staining. Therefore, taken together, VitD3 may have variable effects on lipid accumulation based on dose, cell type and duration of exposure.

Long term survival assay was used to investigate the effect of chronic exposure of VitD3 on melanoma cancer cells. After 10 days treatment with exogenous 10µM VitD3 and sera from mice on CD, there was a significant reduction in cell viability compared to cells exposed to 1µM VitD3 and CD sera. Furthermore, there was a significant decrease in cell activity in B16-F10 cultured with 10µM VitD3 and HFD derived sera in contrast to cells cultured with 1µM VitD3 and HFD sera. These findings are consistent with a report showing human melanoma cell proliferation was strongly inhibited in the presence of VitD3 at 20 ng/mL concentrations after 9 days incubation (Spath *et al.*, 2017). Accumulating evidence supports the notion that inflammation contributes to the progression of various types of cancers (Allavena *et al.*, 2008). Inflammatory mediators such as cytokines promote tumorigenesis through the activation of multiple signalling pathways in the tumour microenvironment (TME) (Mantovani *et al.*, 2008). Recent research suggests that Vitamin D3 can reduce the production of pro-inflammatory cytokines and thereby induce beneficial effects in several cancers (Krishnan and Feldman, 2011). Furthermore, in conditions of metabolic stress with a high-glucose or high-fat diet, vitamin D3 has been found to promote M2-switching and increase the expression of anti-inflammatory cytokines such as IL-10 (Chirumbolo *et al.*, 2017). Added recombinant IL-10 to HFD and VitD3 derived sera significantly inhibited mitochondrial cell activity of a mouse skin cancer cell line (B16-F10) compared to groups treated with sera from HFD only.

The ability of VitD3 to attenuate the production of pro-inflammatory cytokines such as IL-6 by inhibiting p38 signalling, demonstrates its important anti-inflammatory effects in cancer cells (Slominski *et al.*, 2017). However, our results using mitochondrial cell activity assay (MTS) showed that cells cultured with a HFD and VitD3 derived serum

in addition to recombinant IL-6 showed significant increase in mitochondrial cell activity of B16-F10.

5.5 Summary

The goal of this study was to characterize the effects of VitD3 on B16-F10 proliferation, migration as well as lipid up take activity. The results demonstrated the anti-proliferative responses of murine melanoma cell (B16-F10) to different doses of Vitamin D3 (1 and 10µm). Vitamin D3 at high dose is able to significantly prevent lipid inclusion preventing its use as a fuel in cancer cells. Interestingly, the *ex vivo* data showed for the first time that mitochondrial cell activity of B16-F10 was significantly enhanced after treatment with serum derived from $LDLR^{-/-}$ mice fed on a HFD supplemented with VitD3. Furthermore, Vitamin D3 did not counteract the action of IL-6 on B16-F10 viability at the doses chosen. Finally, mouse skin cancer (B16-F10) cell migration was unaffected after treatment with sera from HFD andVitD3 fed mice compared to those treated with HFD alone.

Chapter 6

The effects of Vitamin D3 on melanoma growth in an obese *LDLR* $^{-/-}$ mouse model

The effects of Vitamin D3 on melanoma growth in an obese *LDLR* $^{-/-}$ mouse model

6.1 Introduction

No study has yet investigated the effect of Vitamin D3 on melanoma cell growth, migration, and the production of inflammatory mediators in obese subjects. During the past decade, the non-classical actions of vitamin D3, namely, immune function modulation and hormone secretion, have received much attention. In particular, the potential role of Vitamin D3 analogues alone or in combination with other anticancer agents for the treatment of different types of cancer (Chiang *et al.*, 2011). The association between vitamin D3 status and the higher incidence of many forms of cancer has suggested that vitamin D3 may play a role in the pathogenesis of these types of cancers (Balistreri *et al.*, 2010).

As skin is a primary organ for vitamin D3 synthesis and metabolism (Bikle, 2010), a possible role of vitamin D3 in different skin cancer types in particular melanoma has been reported (Ombra *et al.*, 2017). Some studies suggest a preventative role of VitD3 in melanoma, whereas results on the relationship between dietary intake of VitD3 and cancer risk are controversial and there is inadequate evidence to suggest that VitD3 supplementation decreases the risk for melanoma (Liu *et al.*, 2018). An analysis of 17 prospective studies linked VitD3 deficiency with increased the incidence of lung cancer; however the overall survival rate was not associated with VitD3 levels (Feng *et al.*, 2017). Furthermore, a combined case-control study and meta-analysis showed a negative association between VitD3 levels and papillary thyroid cancer risk (Young and Xiong, 2018). VitD3 can also indirectly prevent cancer, patients who failed treatment to eradicate Helicobacter pylori, which is associated with gastric cancer, had a lower concentration of VitD3 compared to those who were successfully treated (Yildirim *et al.*, 2017). Men with prostate cancer had increased in inflammatory cytokines concentrations and reduced VitD3 levels compared to healthy controls (Xie *et al.*, 2017).

Similarly, inflammatory mediators were elevated in colorectal cancer patients who have VitD3 deficiency (Väyrynen *et al.*, 2016). These observations indicate that indirect anti-cancer actions of vitamin D3 can also be due to its anti-inflammatory properties

Population-based studies, as well as molecular studies, have indicated that VitD3 is implicated in many immune-related diseases such as asthma, atherosclerosis and obesity (Wobke *et al.*, 2014). A retrospective study showed that serum VitD3 levels were negatively correlated with important cardiovascular disease risk factors, such as hypertension, insulin resistance, obesity, and high serum TG levels (Martins *et al.*, 2007). However, Yiu *et al.* (2013), found that oral supplementation of VitD3 for 12 week did not significantly affect vascular function or serum biomarkers of inflammation and oxidative stress. Furthermore, severe and uncontrolled asthma was reversely correlated with VitD3 insufficiency and deficiency (Korn *et al.*, 2013). Finally, published study revealed that lower VitD3 may increase the risk of arteriosclerosis and endothelial dysfunction (London *et al.*, 2007).

VitD3 plays an influential role in the modulation of the inflammatory system by regulating the secretion of cytokines and immune cells, which are essential for the pathogenesis of various immune diseases (**Figure 6-1**) (Liu *et al.*, 2018). Mechanistic studies have revealed that VitD3 impacts on inflammatory processes involved in cancer development, including cytokines and immune cells (Cai *et al.*, 2015). Cytokines release and immune cells infiltration within the inflammatory microenvironment basically serves as direct growth and migratory factors for cancerous cells (Chen *et al.*, 2016; Ariztia *et al.*, 2006).

Among these immune cells, T-regs are a major obstacle to effective anti-tumour responses and contribute to the development of an immunosuppressive tumour microenvironment (TME) (Ombra *et al.*, 2017). Recently, clinical data and animal experiments identified the important role of vitamin D3 in the activity of T regulatory cells (Palmer *et al.*, 2011). VitD3 mediates its action through nuclear receptors (VDR) (Campbell *et al.*, 2010). Most cell types express VDR; however, this expression was negatively associated with tumour differentiation and progression in different cancer types (Jeon and Shin, 2018).

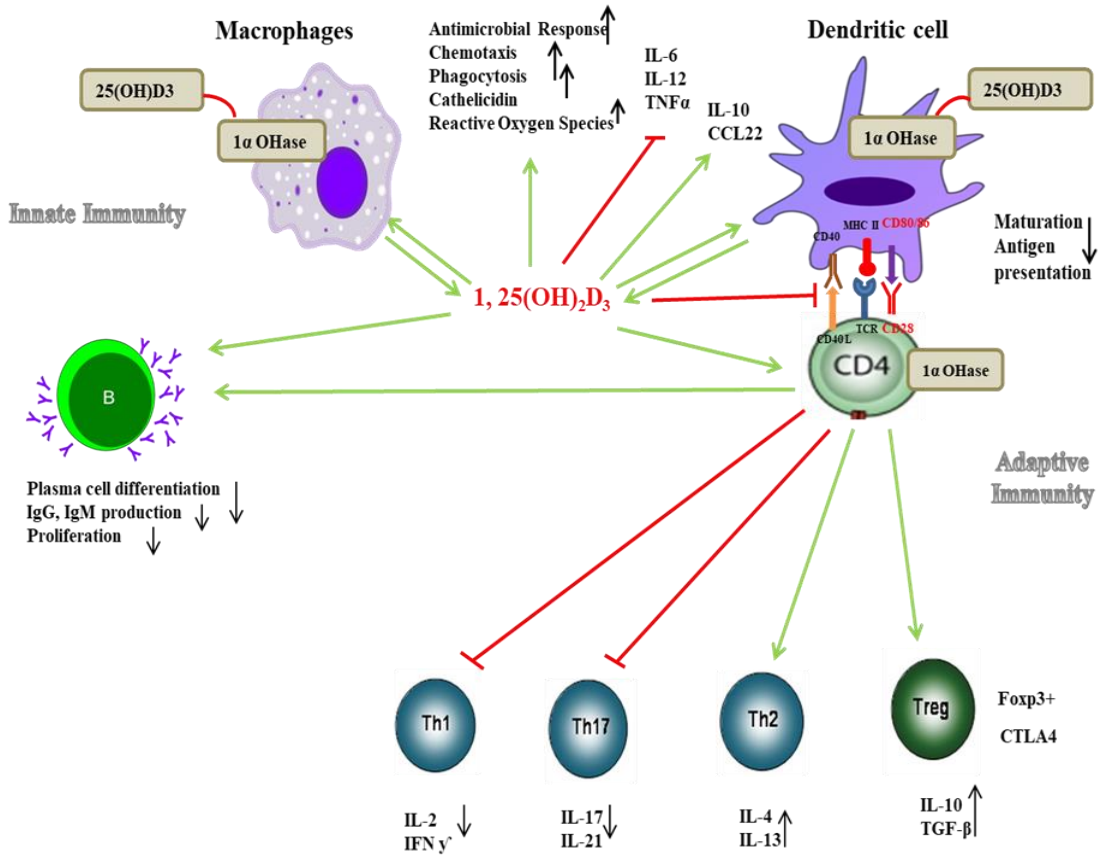


Figure 6-1*: Proposed model of effects of Vitamin D3 on immune responses*.
Vitamin D3 enhances phagocytosis, chemotaxis, production of antimicrobial peptides macrophages differentiation and inhibit dendritic cells (DCs) maturation.

6.2 Chapter aims

This chapter aims to investigate the anti-inflammatory action of Vitamin D3 on melanoma tumour weight and size, its modulatory effect on the tumour microenvironment, changes on cytokines secretion (IL-6, TNF-α, Leptin and IL-10) and chemokine (CCL2) in obese *LDLR*$^{-/-}$ melanoma mouse model.

6.3 Experimental design

Animal experimentation was carried out in accordance with UK Home Office regulations, and institutional ethical guidelines. *LDLR*$^{-/-}$ mice (8 females and 8 males) were randomized into two groups and fed a cholesterol free diet 58R3 (High Fat Diet) (HFD) (Test Diet ® product 20% protein, 36% fat, 35% carbohydrate, rich in sucrose) differing in the content of admixed Vitamin D3(VitD3) (1 IU/g vs 10 IU/g) (Test Diet ® product) for 10-weeks post tumour implantation. Body weight was measured weekly, and after 10-weeks feeding, both groups were subcutaneously injected with 5×10^5 B16-F10 cells and monitored daily for the presence of palpable tumours. After 10 -12 days all mice were terminally anaesthetised, and their spleen and sera collected for analysis (**Figure 6-2**). All mice were age matched. As previous studies reported that Vitamin D3 intake has been lower in obese men, but not in women (Vanlint, 2013a), gender differences were considered.

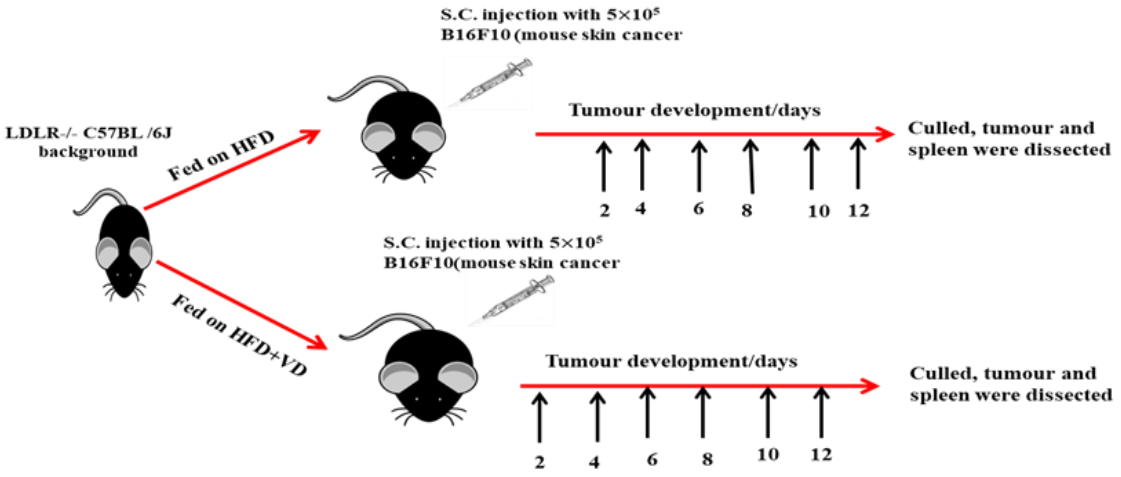


Figure 6-2: schematic of animal experimentation

6.4 Results

6.4.1 VitD3 supplementation has no effect on weight gain in obese *LDLR*$^{-/-}$ tumour bearing mice

The purpose behind measuring body weight was to investigate whether VitD3 induced changes on weight gain after 12-weeks consumption, based on literature Vitamin D3 consumption has impact on the body weight of obese people (Vanlint, 2013a). Therefore, *LDLR*$^{-/-}$ mice were fed on the HFD (with or without VitD3 supplementation) for 10-weeks prior to injection of 5×10^5 B16-F10, and another 2-weeks post tumour implantation.The measurements of body weight were recorded weekly and the percentage weight change was calculated. The weekly measurement indicated a gradual increase in body weight over time feeding HFD plus VitD3 as shown in (**Figure 6-3D**). Our results also showed no significant difference in the weight gain between test groups (P = 0.4837) (**Figure 6-3A**). Similarly males fed on HFD + VitD3 or HFD showed nearly the same body weight (10.13 ± 1.12 *vs*11.73 ± 0.85) (**Figure 6-3C**), while no significant differences was seen between the females group (**Figure 6-3B**). *LDLR*$^{-/-}$ tumour bearing mice fed on HFD + VitD3, and HFD had nearly the same accumulation in abdominal fat deposition after 12-weeks (**Figure 6-4**).

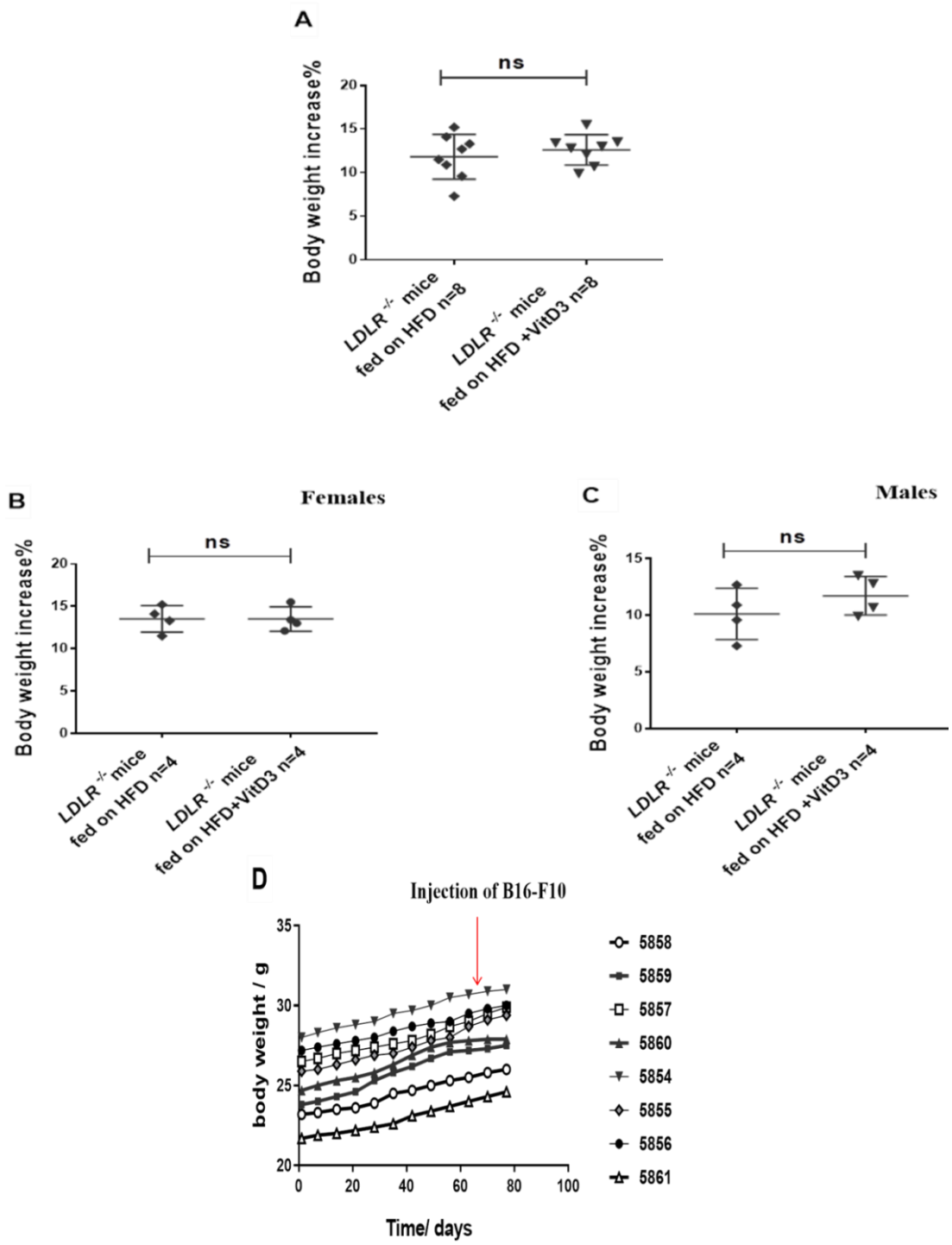


Figure 6-3: Impact of VitD3 supplementation on weight gain in *LDLR*$^{-/-}$ tumour bearing mice.

(**A**) Measurements of body weight percentage in *LDLR*$^{-/-}$ tumour bearing mice following HFD and HFD + VitD3 feeding. The body weight percentage in females and males group respectively (**B** and **C**). (**D**) The weekly changes in weight gain of *LDLR*$^{-/-}$ tumour bearing mice consumed fatty diet supplemented with VitD3.The data are presented as means ±SD and the statistical analysis was performed by unpaired *t* test. ($^{*}p$ <0.05).

* The numbers represent the internal ID numbers of the mice.

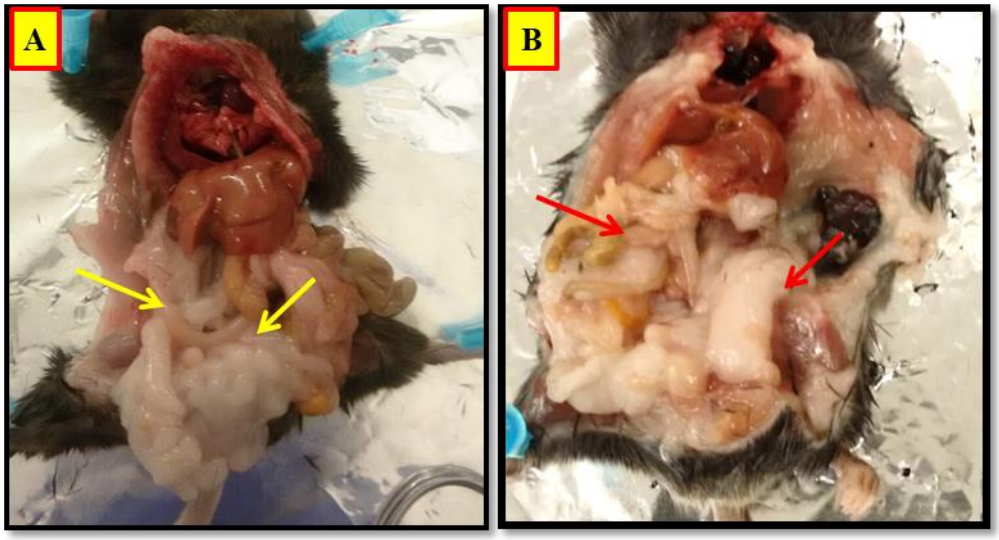


Figure 6-4: Representative pictures of *LDLR*$^{-/-}$ tumour bearing mice after 12-weeks feeding HFD or HFD plusVitD3.
Picture (**A**) Abdominal fat deposition as indicated in yellow arrows in HFD mice. Picture (**B**) Increased in body size and red arrows indicated accumulation of abdominal fat after 12-weeks consuming diet rich in fat plusVitD3.

6.4.2 Characterization of melanoma tumours implanted in *LDLR*$^{-/-}$ mice fed a high fat diet supplemented with VitD3

6.4.2.1 Macroscopic observation of tumour

Macroscopic examination showed that melanoma tumours implanted in mice fed HFD and VitD3 had the same characteristics (HFD chapter 5). Surprisingly, the tumour mass dissected from mice fed on HFD + VitD3 were embedded in thick adipose tissue layer (**Figure 6-5C and D**) compared to fat surrounding tumours in mice fed on HFD only (**Figure 6-5A and B**). Notably, no evidence for melanocytic lymph nodes, no necrosis and no metastatic spread. The injection site was visually checked for infiltration of tumour and vessel formation after extracting the tumour masses. Tumour weight and volume were measured.

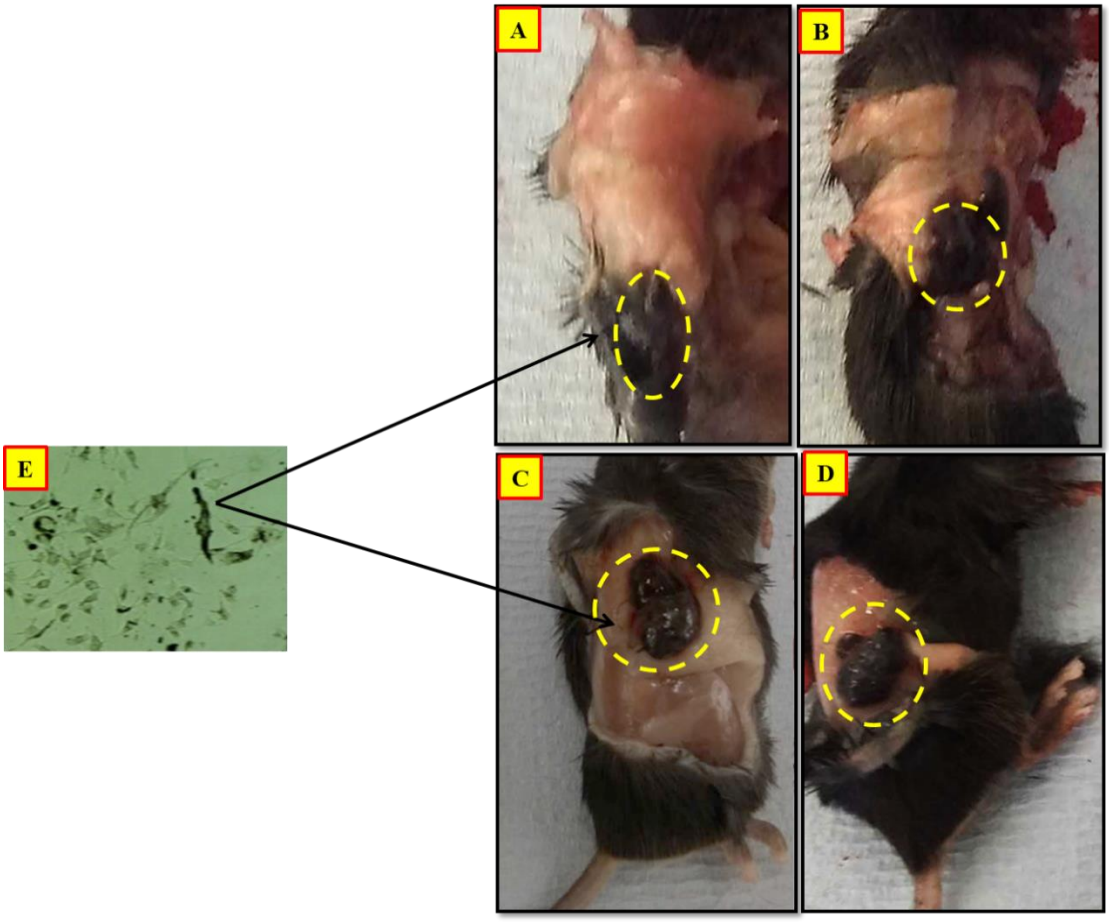


Figure 6-5: Representative images of melanoma tumour implanted in *LDLR*$^{-/-}$ mice fed on HFD plus VitD3 or HFD for 12-weeks.
(**A** and **B**) subcutaneous melanoma tumours in *LDLR*$^{-/-}$ mice fed a HFD .While, photos (**C** and **D**) shows increased in tumour size and embedded in thick fat layer after 12-weeks feeding HFD supplemented with VitD3.

6.4.2.2 VitD3 supplementation increases tumour weight in obese *LDLR*$^{-/-}$ mice

To determine whether a HFD with VitD3 affected tumour growth after 12-weeks feeding compared to HFD only. The measurement of tumour weight showed that VitD3 supplemented HFD had significantly increased melanoma tumour growth compared to their counterparts fed on a HFD alone (P= 0.0013) (**Figure 6-6A**). Furthermore, the data also revealed that females fed on HFD plus VitD3 had heavier tumours compared to the male group fed the same diet (345 ± 20.96 *vs* 273.5 ± 25.21) (**Figure 6-6 B and C**).

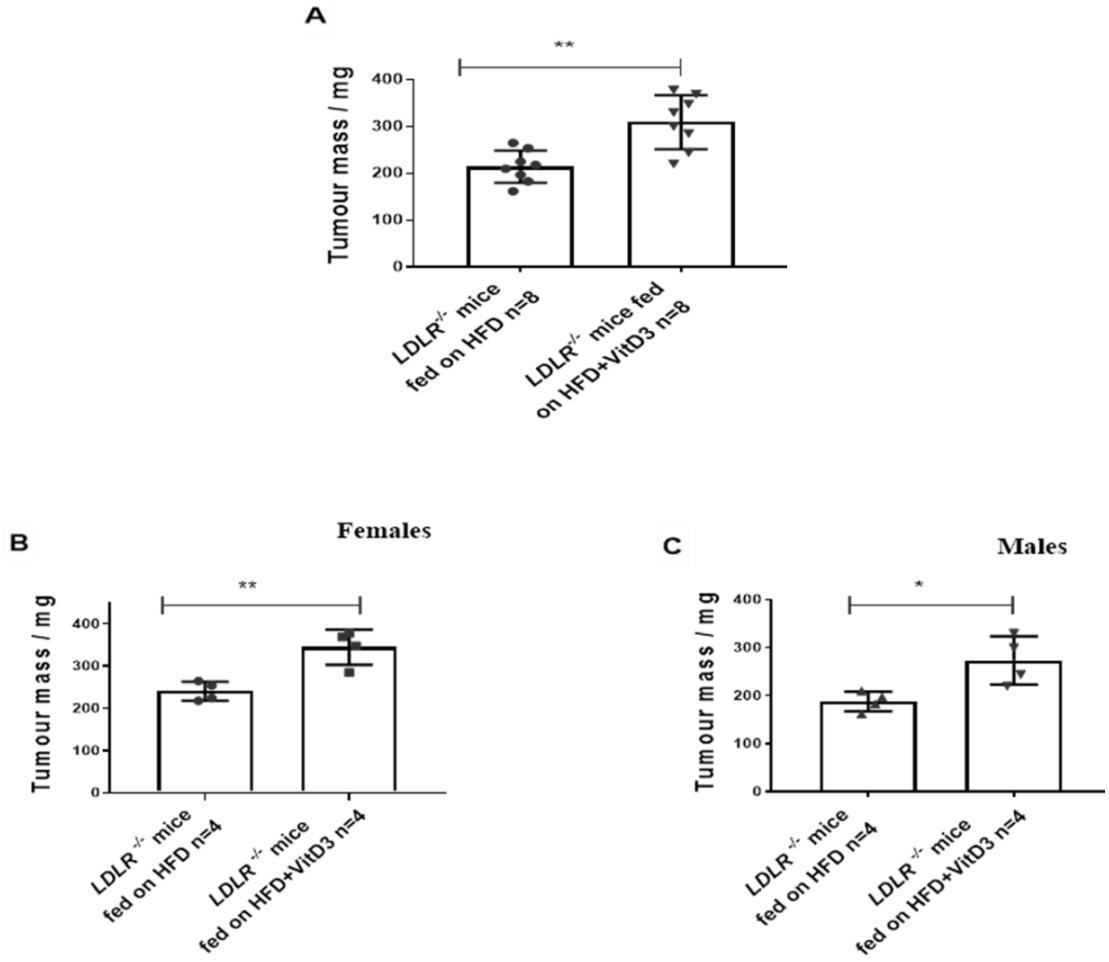


Figure 6-6: Effect of VitD3 supplementation on melanoma tumours weight in *LDLR* $^{-/-}$ mice.

(**A**) Measurements of tumour mass in *LDLR*$^{-/-}$ mice. While (**B** and **C**) Tumour mass in females (n=4) and males (n=4), respectively. Values are expressed as mean ± SD and statistical analysis was performed by unpaired *t* test ($^{*}p$ <0.05).

6.4.2.3 VitD3 supplementation accelerates melanoma tumour volume in *LDLR*$^{-/-}$ mouse model

To compare the volume of tumours in *LDLR*$^{-/-}$ mice after 12-weeks consumption of HFD supplemented with VitD3 and mice fed on a HFD alone, the volume of tumours was measured directly *ex vivo* using the same formula used in previous work (chapter 5). Mice fed on a HFD and VitD3 had significantly larger tumours compared to those fed on HFD alone (p= 0.0008) (**Figure 6-7A**). Tumour volume was also higher in females compared to males (1057 ± 58.34 *vs* 827.8 ± 88.47) (**Figure 6-7B and C**).

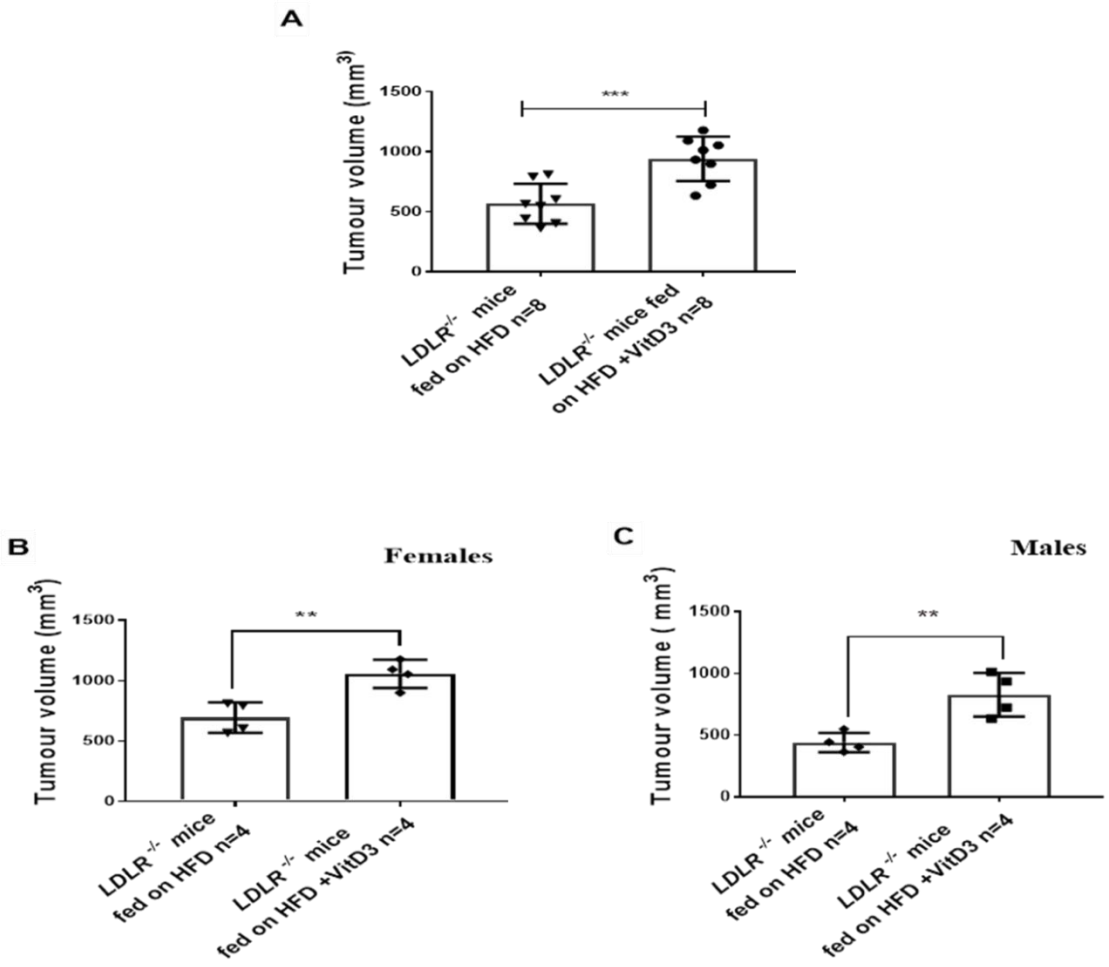


Figure 6-7: The effect of HFD supplemented with VitD3 on tumour volume in *LDLR*$^{-/-}$ mice.

(**A**) Measurements of tumour volume in *LDLR*$^{-/-}$ mice as a whole and split according to sexes (**B** and **C**). Values are expressed as mean ± SD and the total number of mice was (8). Statistical analysis was performed by unpaired *t* test ($^{*}p$ <0.05).

6.4.3 Significant reduction in T-regs accumulation in the spleen of *LDLR*$^{-/-}$ tumour bearing mice fed on VitD3 supplemented diet

Diet-derived nutrients can affect inflammation and immune regulation (Kim and Lee, 2013). Whilst these studies suggest the role of certain dietary components in modulating immunity, a number of questions including whether the effects of dietary components are achievable within the range of physiological concentrations remain unanswered. Evidence of the effect of dietary components on diseases in which T-regs play an influential role may give insight into the role of these dietary components on T-regs function (Issazadeh-Navikas *et al.*, 2012).

Therefore, the effect of 12-weeks dietary consumption of a HFD supplemented with VitD3 on T-regs accumulation in the spleen of *LDLR*$^{-/-}$ tumour bearing mice was analysed by flow cytometry. There was a significant reduction in the frequency of T-regs populations in the spleen of *LDLR*$^{-/-}$ tumour bearing mice (p <0.0001) (**Figure 6-8 A**). Interestingly, the study also found that the reduction in T-regs frequency was slightly higher in males compared to females (11.55 ± 0.89 *vs*13.55 ± 0.49) (**Figure 6-8 B and C**).Three marker were used to distinguish T-regs population ($CD25^+$, $CD4^+$ and $FOXP3^+$) as shown in figure (**6-9**).

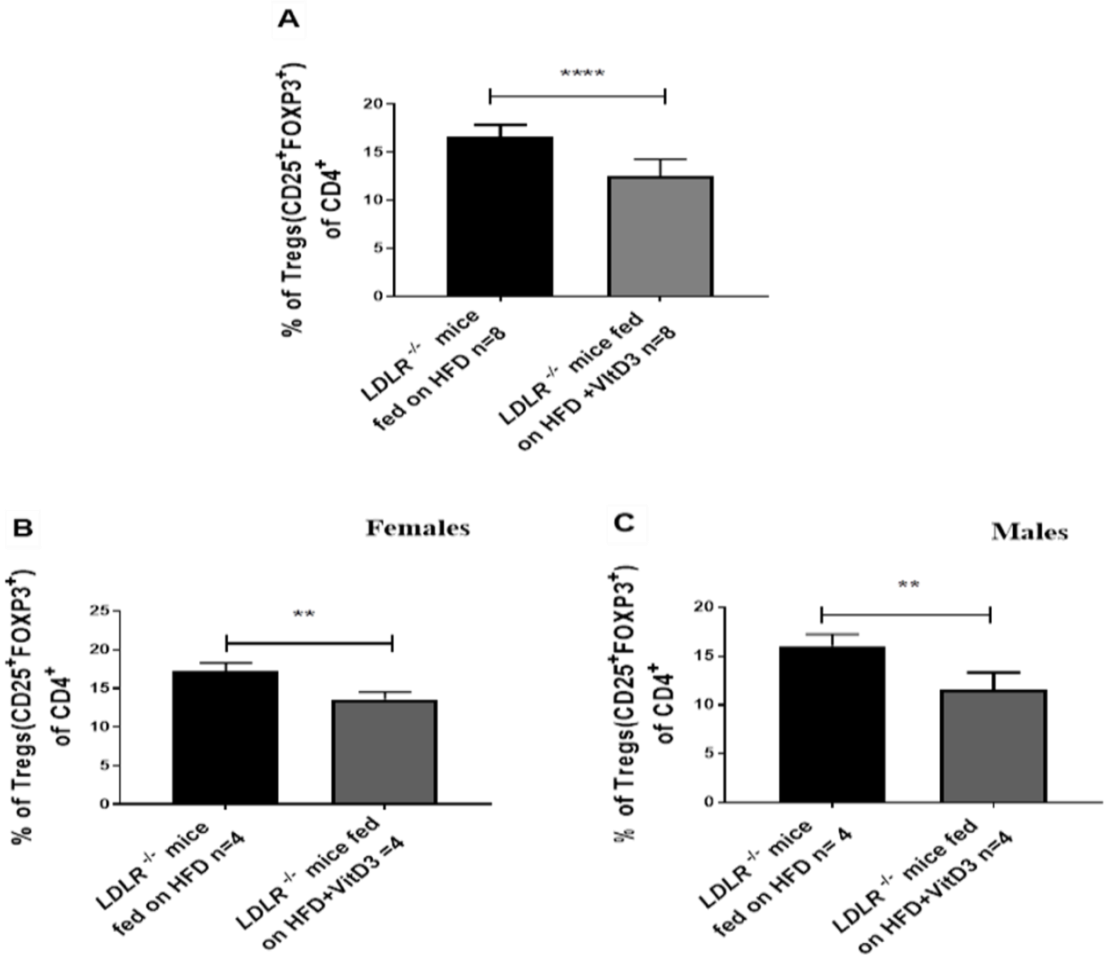


Figure 6-8: Flow cytometry analysis for T-regs population in spleen of *LDLR*$^{-/-}$ tumour bearing mice fed on HFD or HFD plusVitD3 for 12-weeks.

(**A**) The effect of fatty Diet supplemented with VitD3 on the percentage of splenic T-regs population in both groups, while, (**B** and **C**) Percentage in females and males group, respectively. FC analysis was carried out using three markers to identify T-regs population ($CD4^+CD25^+FOXP3^+$). The data are presented as means ± SEM and statistical analysis was performed by unpaired *t* test. ($^*p$ <0.05).

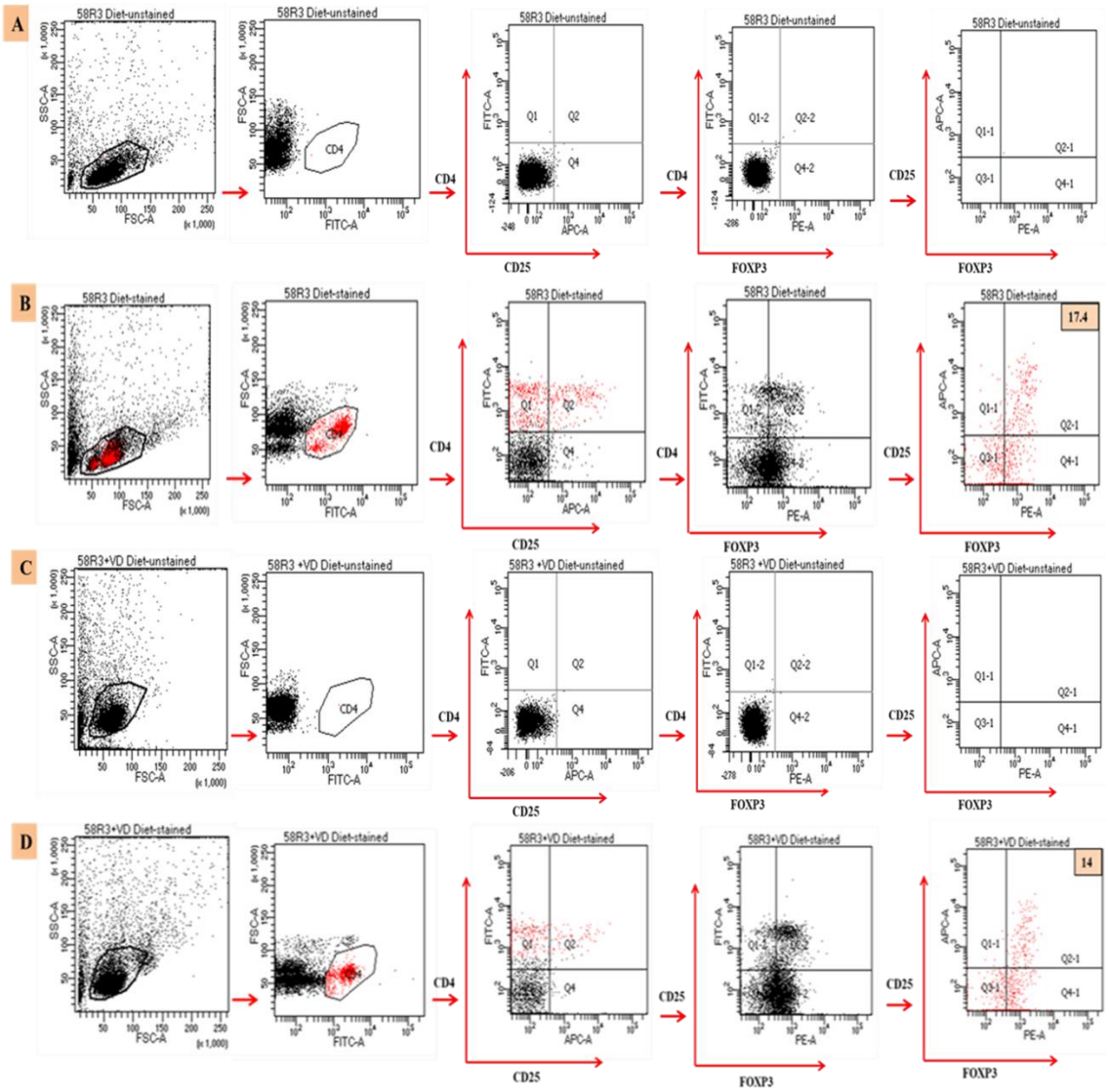


Figure 6-9: Representative dot plots showing percentage of T-regulatory ($CD25^{+}CD4^{+}FOXP3^{+}$) population in the spleen of *LDLR*$^{-/-}$ tumour bearing mice fed on HFD or HFD plus VitD3 for 12 weeks.

FC analysis was done using three markers; CD25, CD4 and FOXP3 to distinguish the T-regs population. These markers were stained with FITC, APC and PE, respectively. The gating was done on $CD4^{+}$ cells and the percentage of Tregs was calculated using, BD FACS Diva TM. (**A** and **C**) dot plots graphs for unstained spleen suspension cell of *LDLR*$^{-/-}$ tumour bearing mice fed on HFD (58R3) and (58R3+VitD3) diet, respectively. While, (**B** and **D**) dot plots graphs for stained spleen suspension cell of *LDLR*$^{-/-}$ tumour bearing mice fed mice fed on HFD (58R3) and (58R3+VitD3) diet, respectively. Numbers in the plots indicate the percent of triple marker positive cell.

6.4.4 The impact of VitD3 supplementation on adipokines levels in *LDLR*$^{-/-}$ tumour bearing mice fed on a HFD and VitD3

Chronic low-grade inflammation (a common obesity features), plays an important role in the aetiology of several diseases such as insulin resistance, cardiovascular disease, and cancers (Mousa *et al.*, 2017). An increasing body of evidence identified the influential role for vitamin D3 in inflammation, and immunoregulation (Hansdottir and Monick, 2011). Several *in vitro* studies showed that VitD3 stimulated production of anti-inflammatory cytokines, inhibited pro-inflammatory cytokines, and regulated immune cell activity (Guillot *et al.*, 2010; Müller *et al.*, 1993). Therefore, the role of a VitD3 supplemented HFD on circulating adipocytokines (Leptin ,TNF-α, IL-6 and chemokine CCL2) was investigated in obese *LDLR*$^{-/-}$ tumour bearing mice.

6.4.4.1.1 The effect of VitD3 supplemented HFD on Leptin levels in *LDLR*$^{-/-}$ tumour bearing mice

Tumour initiation and progression can be affected by chronic inflammation induced by obesity (Lang and Ratke, 2009). Leptin, a hormone secreted in proportion to body fat mass, induces production of pro-inflammatory cytokines (TNF-α and IL-6) by macrophages (Dutta *et al.*, 2012). Previous data found no significant difference in body weight between obese *LDLR*$^{-/-}$ tumour bearing mice fed on VitD3 supplemented HFD or HFD only. The anti- inflammatory effect of a VitD3 supplemented diet on circulating Leptin levels in sera from obese tumour bearing mice was examined. The data found no significant differences in Leptin hormones between mice fed on HFD and those fed on HFD supplemented with VitD3 (**Figure 6-10A**). Furthermore, the results also showed no significant differences between gender groups (**Figure 6-10B and C**).

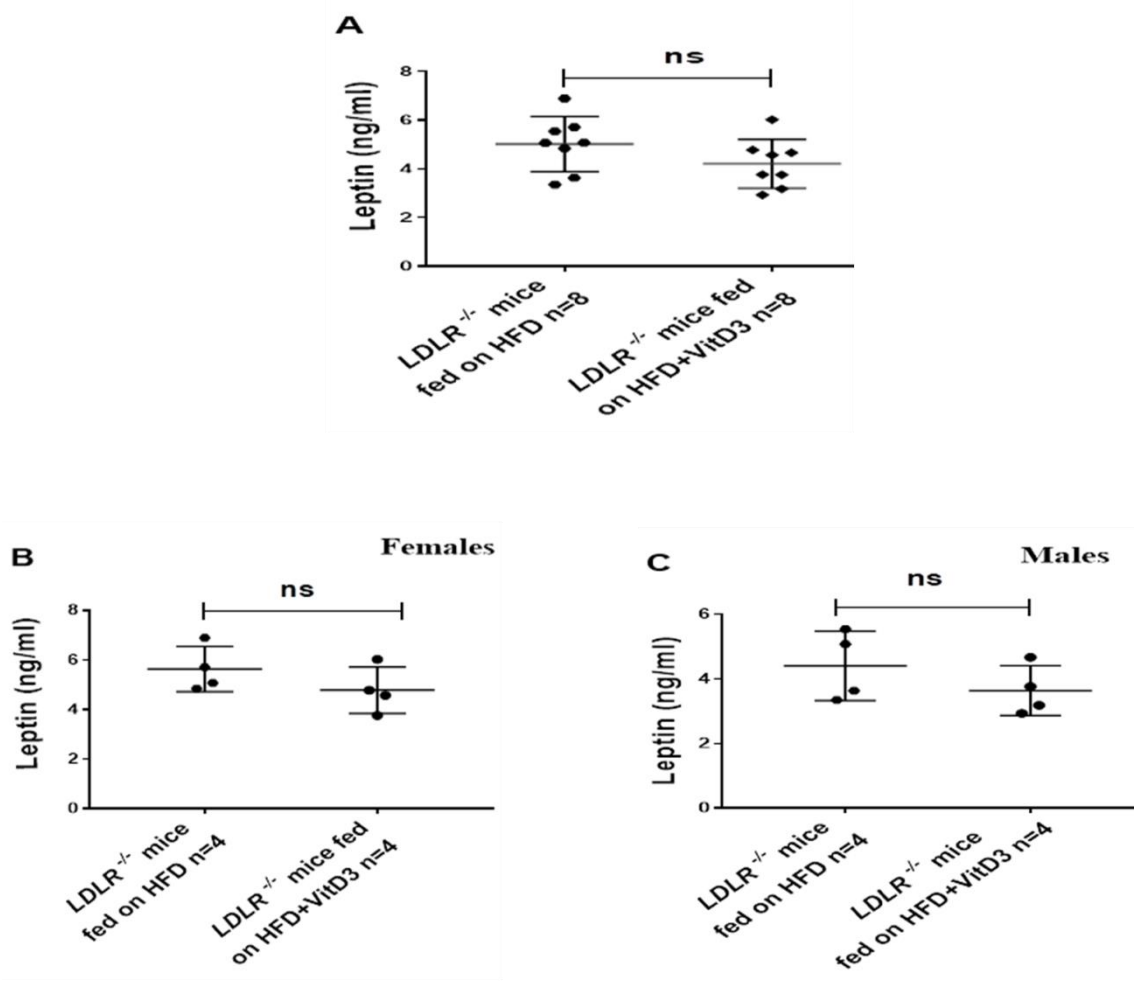


Figure 6-10*:* ELISA measurements of Leptin protein expression in *LDLR*$^{-/-}$ mice fed on fatty diet supplemented with VitD3 or HFD.

(**A**) The levels of serum Leptin in mice fed with HFD plus VitD3 or HFD for 12-weeks. (**B** and **C**) levels in females and males group, respectively. The data are represented means ±SEM from triplicate measurement (n=3). Statistical analysis was performed by unpaired *t* test. ($^{*}p < 0.05$).

6.4.4.1.2 The effect of VitaminD3 supplemented HFD on CCL2 levels in serum from *LDLR*$^{-/-}$ tumour bearing mice

Elevated CCL2 levels in the tumour microenvironment (TME) and blood circulation are associated with poor prognosis in several cancers (Li *et al.*, 2013).Thus, the impact of VitD3 supplementation on the circulating chemokine CCL2 was investigated in the sera of tumour bearing *LDLR*$^{-/-}$ mice using ELISA. There was no significant difference in the secretion of CCL2 in the sera of mice fed on HFD plus VitD3 and those fed on the HFD alone (**Figure 6-11A**). The results also showed that VitD3 has no effect on CCL2 secretion in both females and males (**Figure 6-11B and C**).

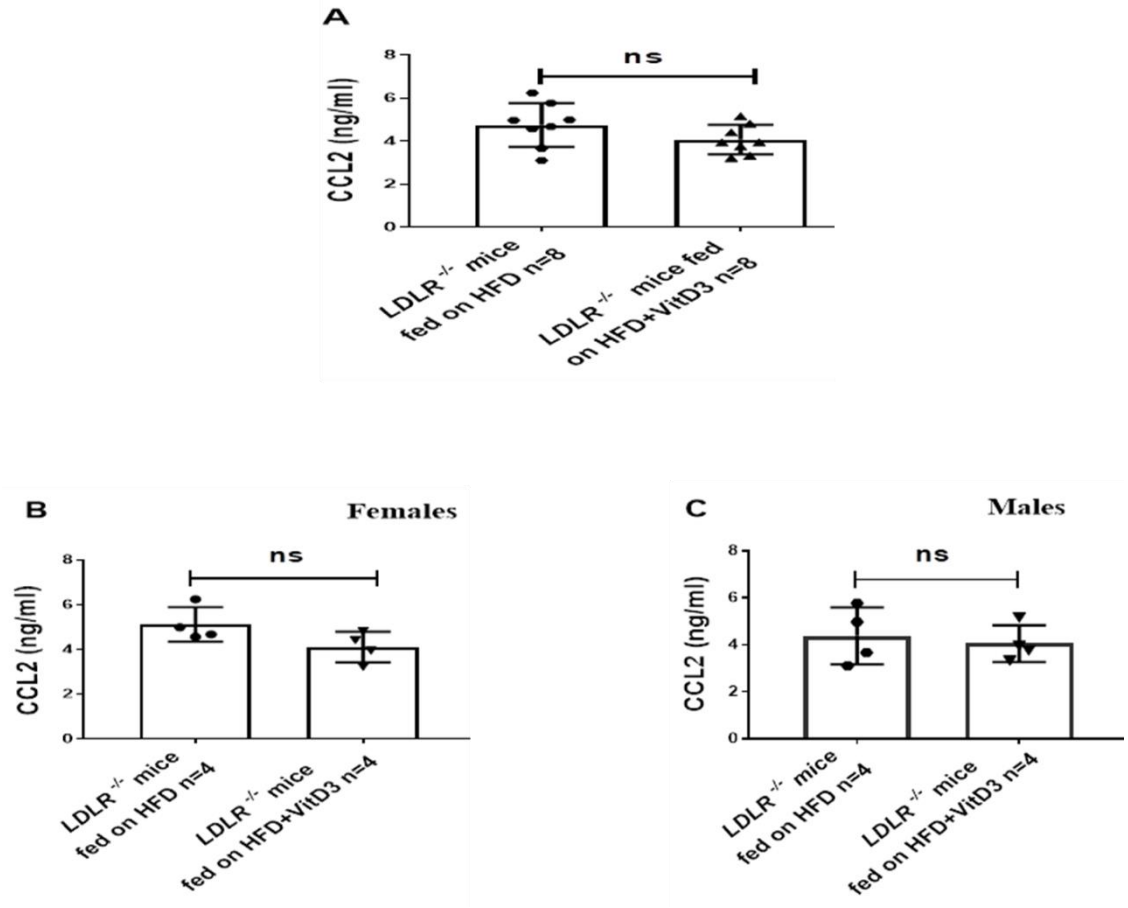


Figure 6-11: Expression of CCL2 protein in sera of obese *LDLR*$^{-/-}$ tumour bearing mice.
(**A**) Concentrations of CCL2 protein in mice sera consumed diet rich in fat plus VitD3 or fatty diet only for 12-weeks. While, (**B** and **C**) levels of circulating CCL2 in females and males sera, respectively. The data are represented means ±SEM from triplicate measurement (n=3). Statistical analysis was performed by unpaired *t* test ($^{*}p < 0.05$).

6.4.4.1.3 The effect of VitaminD3 supplemented HFD on TNF-α levels in *LDLR*$^{-/-}$ tumour bearing mice

Tumour necrosis factor α (TNF-α), a potent inflammatory biomarker is elevated in the periphery during systemic inflammation (Calton *et al.*, 2015). Thus, we examined the effect of VitD3 as an anti-inflammatory agent on TNF-α concentration in sera of *LDLR*$^{-/-}$tumour bearing mice using ELISA. There was no significant difference in TNF-α levels in the presence or absences of VitD3 (**Figure 6-12A**). Furthermore, ELISA measurements also found no significant difference in TNF-α secretion between ganders (**Figure 6-12 B and C)**.

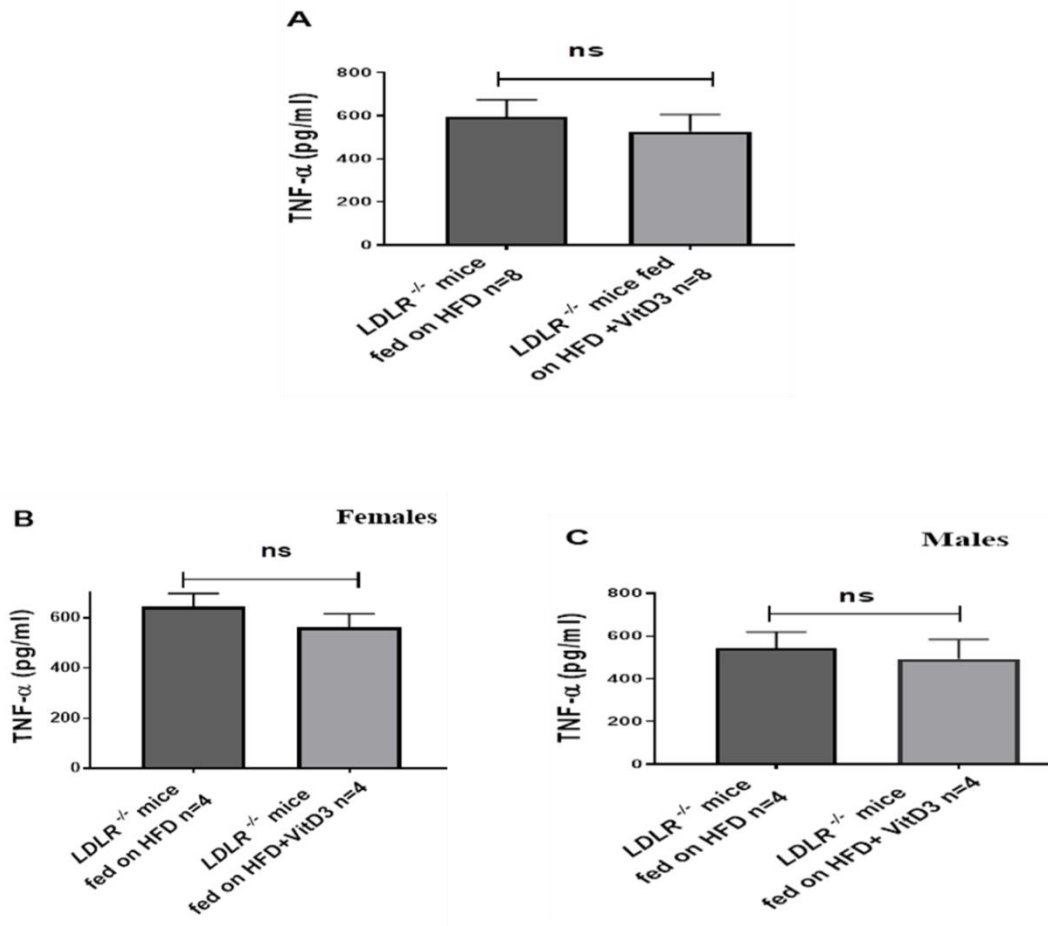


Figure 6-12: Quantitative measurements of TNF-α in *LDLR*$^{-/-}$ tumour bearing mice sera fed on HFD or HFD + VitD3.

(**A**) Concentrations of TNF-α in serum of mice fed with a HFD plus VitD3 or HFD for 12-weeks. While, (**B** and **C**) levels of TNF-**α** in females and males sera, respectively. The data are represented means ± SEM from triplicate measurements (n=3). Statistical analysis was performed by unpaired *t* test ($^{*}p < 0.05$).

6.4.4.1.4 The impact of VitD3 supplemented diet on circulating IL-6 levels in *LDLR*$^{-/-}$ tumour bearing mice

Among pro-inflammatory cytokines, Interleukin-6 (IL-6) has emerged as an important player in tumour-associated inflammation (Grivennikov and Karin, 2011). High levels of IL-6 in the circulation may contribute to adipose tissue expansion in obesity, as adipocytes are an important producer of this cytokine (Sindhu *et al.*, 2015). Thus, we hypothesized that VitD3 supplementation may decrease the secretion levels of pro-inflammatory IL-6 in the sera of tumour bearing mice. The results showed no significant difference found between tested groups (**Figure 6-13A**). There is no effect of VitD3 on IL-6 levels between genders (**Figure 6-13 B and C**).

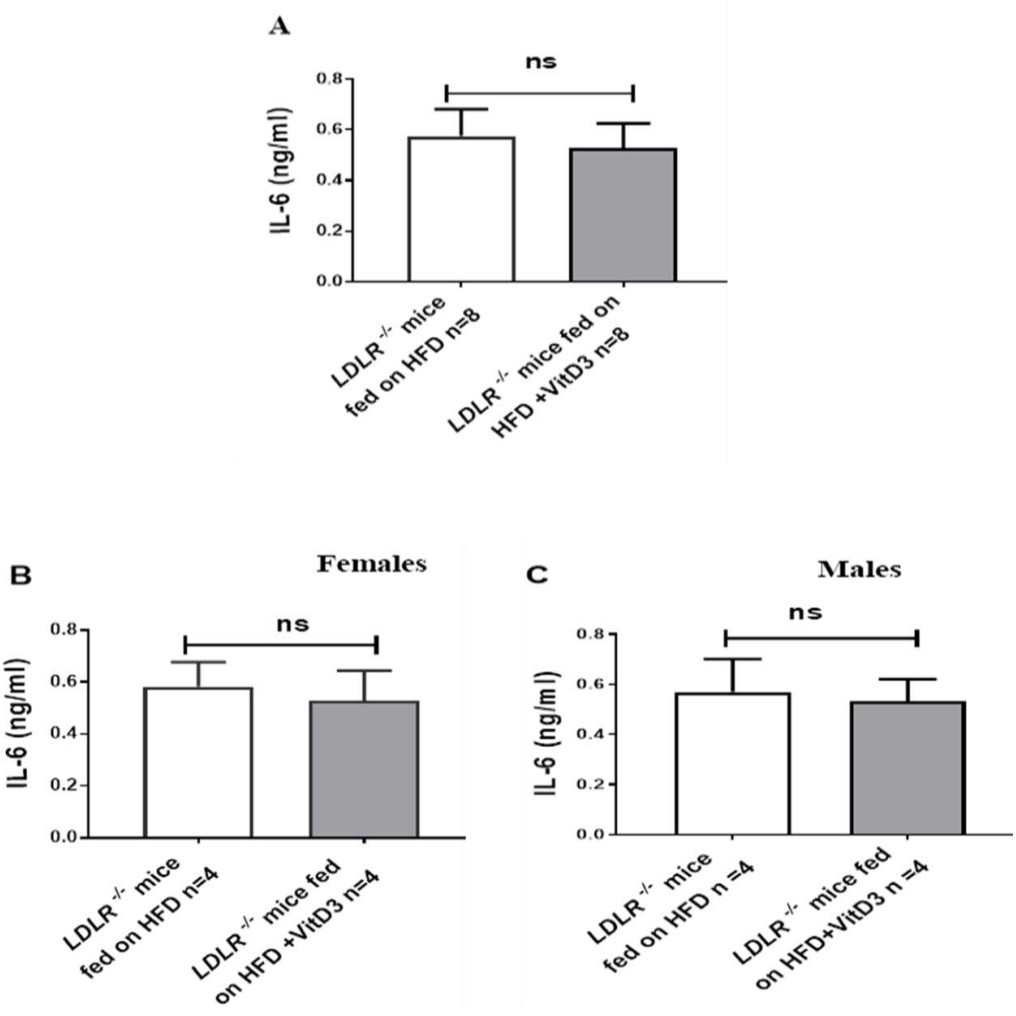


Figure 6-13: ELISA measurements of circulating IL-6 in serum of *LDLR*$^{-/-}$ tumour bearing mice fed on VitD3 supplemented HFD or HFD.

(**A**) Concentrations of IL-6 protein in mice sera consumed HFD plus VitD3 or HFD only for 12-weeks. (**B** and **C**) IL-6 protein expression in females and males serum, respectively. The data are represented means ± SEM from triplicate measurements (n=3). Statistical analysis was performed by unpaired *t* test. ($^{*}p < 0.05$).

6.4.4.1.5 The effect of VitD3 supplementation on IL-10 concentration in *LDLR*$^{-/-}$ tumour bearing mice

Several Publications reported a positive correlation between elevated plasma levels of anti-inflammatory cytokine IL-10 and insulin sensitivity in healthy subjects and reduced in obese and diabetic subjects (Hong *et al.*, 2009; Charles *et al.*, 2011). IL-10 is a main inhibitor cytokine which is has the ability to suppress macrophage functions and inhibits the secretion of pro-inflammatory cytokines (Pontillo *et al.*, 2003). Therefore, the effect of VitD3 supplementation on IL-10 cytokine was investigated in obese *LDLR*$^{-/-}$ tumour bearing mice. There was no significant difference in the levels of IL-10 in sera of mice fed on HFD supplemented with VitD3 compared to those fed on HFD alone (**Figure 6-14A**). Furthermore, there were no difference in the levels of IL-10 between sexes (**Figure 6-14 B and C**).

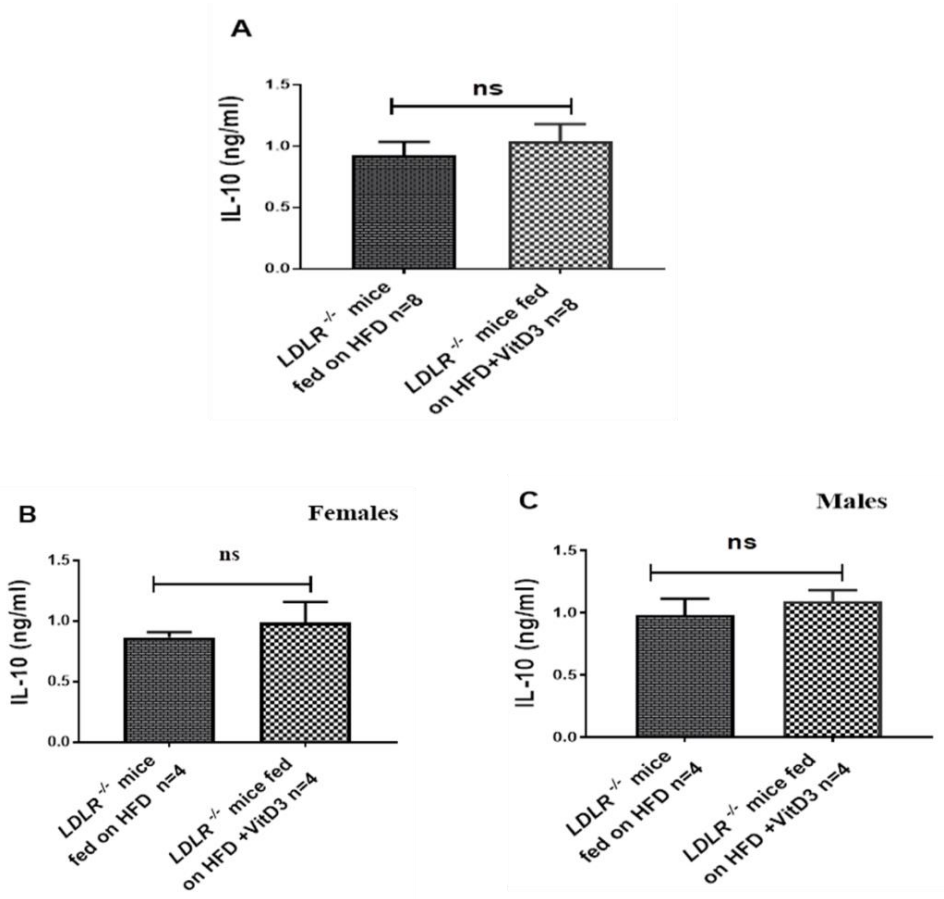


Figure 6-14*:* Quantitative measurements of IL-10 in *LDLR*$^{-/-}$ tumour bearing mice serum fed on VitD3 supplemented HFD or HFD.

(**A**) IL-10 protein in mice serum fed on fatty diet plus VitD3 or HFD only for 12-weeks. While, (**B** and **C**) The levels of circulating cytokine IL-10 in females and males sera, respectively. The data are represented means ± SEM from triplicate measurements (n=3). Statistical analysis was performed by unpaired *t* test ($^{*}p < 0.05$).

6.5 Discussion

In addition, to the traditional role of Vitamin D3 in maintaining healthy mineralisation of bone, VitD3 also plays an important role in inflammation and immunoregulation. Low VitD3 levels have been implicated in the pathophysiology of various inflammatory diseases and conditions associated with chronic low-grade inflammation, such as obesity, insulin resistance and type 2 diabetes (Mousa *et al.*, 2017). There is an inverse association between Vitamin D3 status and changes in BMI (Mehmood and Papandreou, 2016). However, the direct mechanism underlying this association remains unclear. Another area of controversy regards the role of Vitamin D3 in cancer, as accumulating data suggested an association between VitD3 deficiency and incidence of various cancers including colorectal, pancreatic, breast, kidney and melanoma cancer (Rogers *et al.*, 2010). It has been found that consumption VitD3 as a dietary supplement may decrease the risk of skin cancer (Park *et al.*, 2016). Furthermore, a study of German patients with melanoma reported that lower serum 25(OH) D3 concentrations are associated with increased risk for melanoma cancer (Bade *et al.*, 2014). However, the association between levels of 25(OH)D3 and melanoma cancer risk remain controversial with other publications reporting no relationship between serum 25(OH)D3 levels and poor prognosis of melanoma (Freedman *et al.*, 2007),or an increased incidence of melanoma in patients with higher levels of vitamin D3 (van der Pols *et al.*, 2013; Newton-Bishop *et al.*, 2015). Importantaly, the levels of this vitamin are affected by other factors including body mass index (BMI), gender, age and season (Lagunova *et al*., 2009).

This study aimed to investigate the role of a Vit D3 supplemented diet on melanoma tumour in a mouse model of obesity. We previously found that tumours appeared earlier and were larger and heavier in the obese mice compared to lean once, showing that HFD diet induced-obesity accelerated melanoma tumour growth as well as progression. Although most available evidence supports the notion that vitamin D3 may attenuate tumour growth, evidence for its possible effect on skin cancer is still limited. Previously, a prospective cohort study of the Danish general population showed that higher levels of VitD3 is associated with increased risk of non-melanoma and melanoma skin cancer (Afzal *et al*., 2013). Similarly, a meta-analysis found that an increase in the VitD3 blood circulation was associated with increased risk of basal cell carcinoma and melanoma (van der Pols *et al.*, 2013).

Interestingly, our findings revealed that melanoma tumour weight and volume were significantly increased in *LDLR*$^{-/-}$ mice after 12-weeks of a HFD supplemented with VitD3. These findings might be attributed to the ability of Vitamin D3 to supress adaptive immune responses (Aranow, 2011). As melanoma is an immunogenic tumour, vitamin D3 supplementation may be potentially harmful for melanoma patients (Field *et al.*, 2013). These findings partially agree with Karkeni *et al.* (2019) who found that female C57BL/6J mice fed on HFD for 8-weeks and received VitD3 (40IU) after tumour implantation for 2-weeks, bearing significantly heavier and bigger breast tumours compared to mice fed on control diet.

Several mechanisms have been suggested to explore how vitamin D3 could affect the inflammatory microenvironment in cancers such as regulating the interaction between immune and tumour cells in tumour microenvironment (TME), regulate the levels of cytokines, inhibiting the *NF-κB* signalling pathway and regulating immune cell activity (Raymond *et al.*, 2014; Han *et al.*, 2015).

Dysregulation of vitamin D3 metabolism and function in cancer cells has recently emerged as a novel explanation for resistance to the anti-tumorigenic action of vitamin D3 and therefore, development and progression of cancer (Jeon and Shin, 2018). Expression of CYP27B1 (an enzyme responsible for transforming calcidiol to calcitriol) is inversely correlated with the progression of different cancers including lung, prostate, colon, parathyroid, and skin (Brozyna *et al.*, 2011; Segersten *et al.*, 2002; Hsu *et al.*, 2011; Matusiak and Benya, 2007; Chen *et al.*, 2003; Mawer *et al.*, 1994; Hansdottir *et al.*, 2008). Interestingly, pro-inflammatory cytokines such as IL-6 and TNF- α have been found to down-regulate CYP27B1expression in colon cancer, which suggested that the pro-inflammatory mediaters in tumour microenvironment may play an important role in decrasing CYP27B1 levels during cancer progression (Hummel *et al*., 2014). Furthermore, another important player in VitD3 metabolism is CYP24A1, an enzyme that degrades calcidiol and calcitriol. Cancerous cells can up regulate CYP24A1 expression to reduce local concentrations of calcitriol and are correlated with the advanced stages of colon, prostate, breast, and lung cancers (Jeon and Shin, 2018).

VitD3 may also work as a pre- survival molecule that helps cells to maintain their bioenergetic and survival homeostasis via modulation of the stress and damage response. This modulation also includes the ability of vitamin D3 to enhance and

improve an organism's clearance of disturbing and stressful agents (Chirumbolo *et al.*, 2017). Murine models and patient-based studies have demonstrated a relationship between VitD3 status and T-regs numbers and/or function (Chambers and Hawrylowicz, 2011). Despite the well-established data indicating that VitD3 increased T-regs and enhanced transcription Foxp3 (+) expression (Gorman *et al.*, 2016), this study's flow cytometry results revealed that splenocytes of obese mice fed on VitD3 supplemented HFD diet had significantly lower $CD4^{+}CD25^{+}FOXP3^{+}$ (T-regs) percentage in spite of these mice bearing heavier tumours compared to their counterpart who have significantly higher T-regs percentage. These findings might be attributed in part of to the infiltration of these cells in the tumour site.

The biologic effects of vitamin D3 are largely correlated with the presence and activity of the vitamin D receptor (VDR), which serves as a nuclear transcription factor and binds to DNA sequences known as vitamin D3 response elements (van Harten-Gerritsen *et al.*, 2015).Vitamin D3 regulates multiple signalling pathways involved in proliferation, apoptosis, differentiation, and angiogenesis, and can therefore influence cancer development and growth (Liu *et al.*, 2018). Certainly, VitD3 induce anti-proliferative activity on melanoma cell lines *in vitro*. However, there was an inability of these lines to respond to the anti-proliferative action of VitD3 when progression occurs due to reduce expression of the VitD3 receptor (Maria *et al*., 2017). Additionally, as a large variety of immune cells such as T and B-lymphocytes, monocytes and DCs express the VDR, Vitamin D3 could modulate the innate and adaptive immune response (Liu *et al.*, 2018). Furthermore, the stimulation of $CD4^{+}$ T cells in APC free medium with vitamin D3 suppresses proliferation capacity, and enhances the expression of PD-L1, and CTLA-4 inhibitory markers on $CD4^{+}$ T cells (Sheikh *et al.*, 2018). Our measurement of body weight indicates no significant changes in weight gain after 12 weeks consumption dietary fat supplemented with VitD3 in both gender. However, there was slight increase in body weight of males fed a HFD plus VitD3 compared to mice fed on HFD only.

Our results agree with Swami *et al*. (2017), who found no significant changes in body weights compared to controls in lean and obese tumour bearing mice consuming VitD3 for 16 weeks in a breast cancer model. Using a mouse model of non-alcoholic fatty liver disease, Kheder *et al*. (2017) also found that $LDLR^{-/-}$ mice did not exhibit significant changes in their body weight gain after 10 weeks consumption of a VitD3 supplemented

HFD compared to those fed on a HFD alone. Furthermore, several clinical trials investigating the impact of increased in vitamin D3 status on body weight gain have not provided a consistent data on the possible effects of vitamin D3 supplementation on weight gain (Barrea *et al.*, 2017). In the context of cancer related inflammation, VitD3 has a regulatory effect on some of the key molecular pathways involved in inflammation (Liu *et al.*, 2018). However, evidence-linking VitD3 with immune responses in tumours is still poorly characterised. Therefore, the effect of a VitD3 supplemented HFD on cytokine and chemokine secretion in obese *LDLR*$^{-/-}$ tumour bearing mice was examined.

Inflammatory responses play a significant role in cancer pathogenesis, including tumour initiation, promotion, and development. Cytokines are now considered as key mediators linking inflammation and cancer (Park *et al.*, 2016). Amongst these cytokines, the pro-tumourogenic capacity of TNF- α and IL-6 is well established. The role of TNF- α and IL-6 as chief regulators of tumour-associated inflammation and tumourigenesis makes them attractive targets in cancer treatment (Taniguchi and Karin, 2014; Grivennikov and Karin, 2011). By ELISA- based methods, our results showed no significant differences in the secretion of pro- inflammatory adipokines secretion (TNF-α, IL-6) respectively in mice sera fed on diet rich in fat supplemented with VitD3 and those fed on high fat diet only. These findings are in agreement with data obtained from meta-analysis study, which is found that vitamin D3 supplementation does not have a significant influence on the concentration of selected inflammatory biomarkers (TNF-α, IL-6) in obese and overweight subjects (Jamka *et al.*, 2016). However, previous studies found that vitamin D3 down-regulated the expression and production of several pro-inflammatory cytokines such as TNF- α, IL-6, and IL-8 in patients with immune-related disease (Calton *et al.*, 2015; Meeker *et al.*, 2014). Another published data found that dietary vitamin D3 decreased inflammatory cell infiltrates and a reduced expression of pro-inflammatory cytokines TNF- α, IL-6 in a mouse model of colon cancer (Meeker *et al.*, 2014). One possible mechanism by which VitD3 down-regulates immune functions is by suppressing nuclear factor-kappa B (NF-κB) (Song *et al.*, 2013). Additionally, VitD3 down-regulated the expression of Toll-like receptors, which may be associated with, decreased production of IL-6, and TNF-α (Khoo *et al.*, 2011).

Leptin an adipose tissue-derived hormone and important player in tumourigenesis (Stattin *et al.*, 2004), induces production of pro-inflammatory cytokines by macrophages, and shifts the balance of T-helper toward a TH1 phenotype in obesity related diseases (Dutta *et al.*, 2012). As this hormone is secreted in proportion to the adipose mass in an individual, we hypothesised that VitD3 as a regulator of energy metabolism may effect Leptin secretion in obese tumour bearing mice. ELISA results showed that VitD3 supplementation had no effect on circulating Leptin levels. This was inconsistent with Rocha *et al.* (2017), who found an increase in fat mass percentage and higher Leptin concentrations in patients with vitamin D3 deficiencies. Furthermore, Leptin secretion was powerfully inhibited in human adipose tissue treated with VitD3 *in vitro* (Koszowska *et al.*, 2014).

Our measurement also found that VitD3 had no effect on the leveles of chemokine CCL2/MCP-1 and anti-inflammatory IL-10 in obese mice sera fed on a diet rich in fat supplemented with VitD3. Our results concur with Mousa *et al.* (2017), who found that VitD3 supplementation had no effect on circulating levels of pro-inflammatory and anti-inflammatory mediators such as CCL2, TNF-α, IL-6 and IL-10 respectively in obese and overweight patients.

6.6 Summary

There was no effect of a VitD3 supplemented HFD on body weight increase after 12 weeks consumption compared with those fed a HFD alone. This indicates the inability of this hormone to prevent or reduce the effect of HFD on weight gain in *LDLR*$^{-/-}$ mice. Interestingly, mice fed on HFD plus VitD3 had heaver, larger tumours compared to their counterpart fed HFD alone. Surprisingly, tumour-bearing mice fed on VitD3 had significantly lower T-reg percentage in their spleen in contrast to those consumed HFD only. Therefore, it seems likely that VitD3 caused an increase in tumour growth because of its anti-inflammatory capacity, in which it shapes cell activity without affecting systemic cytokine levels that we tested.

Chapter 7

Concluding Discussion

Limitations

Future directions

Concluding Discussion
Limitations and future directions

7.1 Concluding Discussion

This thesis investigates three main aspects, firstly, the influence of fatty diet induced obesity on melanoma tumour growth and their alterations on the tumour microenvironment (TME) in mice with a genetic deletion in the low density lipoprotein receptor *(LDLR$^{-/-}$)* on a C57BL/6 background as a model of hyperlipidaemia. Secondly, we identified the role of a VitD3 supplemented HFD on tumour growth in obese mice using a *(LDLR$^{-/-}$)* melanoma mouse model. Finally, we assessed the impact of these diets on splenic T-regulatory (T-regs) frequency of *LDLR$^{-/-}$* tumour bearing mice.

Before establishing *in vivo* work, in *vitro* experiments were done to model the interaction of two chief players within TME, namely the tumour cells and tumour resident macrophages (using J774 macrophage and B16-F10 melanoma cell lines). The action of immune modulators was also examined including fatty acids (Oleic +palmitic acids 2:1) and serum derived from mice fed on a HFD or CD. The expression of functional receptors and phenotypic and tumorigenic actives of these cells was examined (chapter 3). The anti-inflammatory action of Vitamin D3 on mitochondrial cell activity, lipid inclusion ability and migration of B16-F10 cells was *in vitro* investigated (chapter5). Importantly, in this thesis the melanoma cancer cell line B16-F10 was cultured in phenol free medium to reduce estrogenic activity of phenol red which may affect the results as phenol red known to have a weak estrogenic activity on cancer cells (Wesierska-Gadek *et al.*, 2007). In order to test our hypothesis *in vivo* animal investigations were carried out using syngeneic implantation of B16-F10 in *LDLR$^{-/-}$* mice fed on HFD, CD and HFD plus VitD3 (chapters 4 and 6).

7.2 Characterisation of HFD induced obesity in a melanoma mouse model

Epidemiological studies and clinical trials showed that fatty diets (i.e. a high caloric diet) induced obesity leads to adipose tissue enlargement increasing the incidence of melanoma and worsening patient prognosis (Chen *et al.*, 2016; Calle *et al.*, 2003). Mouse melanoma models also show that a diet rich in fat, induced obesity and promoted melanoma growth and progression (Malvi *et al.*, 2015; Pandey *et al.*, 2012; Jung *et al.*, 2015). Malignant melanoma cells grow in the anatomical vicinity of adipose tissue, suggesting that adipocytes may provide energy for rapid tumour cell proliferation (Kwan *et al.*, 2014).

All these studies provide a firm basis for an association between obesity and melanoma incidence and occurrence, suggesting that strategies to control obesity may be a promising target for reducing the risk of melanoma. Murine melanoma models have been commonly used to broaden our understanding of tumour pathogenesis, as these models simulate natural tumour progression, from proliferation to invasion and metastasis. Cancer cells exist as diverse entities and the outcome of malignancy is significantly determined by the crosstalk of cancer cells with the surrounding microenvironment. Additionally, data generated from these models allow a better understanding of the interaction between the tumour microenvironment (TME) including ongoing immune responses. Accordingly, the results obtained from these models my lead to the development of novel immune modulators (Kuzu *et al.*, 2015).

Models of diet-induced obese animals/diet-induced obesity (DIO) have proven invaluable in the basic science of the diseases by identifying the role of chronic inflammation, and metabolic complication (Islam and du Loots, 2009). Diets rich in fat are commonly used to induce obesity in murine and other rodent models. A HFD used to induce obesity models and chronic inflammation, which is an important pathogenic mechanism in several diseases including cancer (Hayashi *et al.*, 2018). In addition, Jung *et al*. (2015) who found that feeding C57BL/6N mice a diet rich (60% kcal) in fat for 16 weeks induced a significant increase in body weight gain in a melanoma mouse model. In another study conducted by Chen and co-workers (2016) who found that 6-week-old male mice feed for 6-weeks on a HFD (60% kcal) had a significant increase in weight

gain with high adipose tissue accumulation. Furthermore, 4–5 week old male C57BL/6J mice fed on a HFD showed a marked increase in weight (Pandey *et al.*, 2012).

It is important to mention, that several aspects should be taken into consideration when designing mouse models of obesity such as duration of the HFD, age and gender of the mice which may have different responses to HFD. Rodent strains and the formulation of the HFD are important as well (Heydemann, 2016). In addition, most common mouse models used for studying obesity and their complications arose from spontaneous mutations. Among these are LDL receptor-deficient (*LDLR*$^{-/-}$), Leptin-deficient (*Lep* $^{ob/ob}$), and leptin receptor-deficient (*LepR* $^{db/db}$) mice (Kennedy *et al*. 2010). Published data found that *LDLR*$^{-/-}$ mice fed on diets rich in fat (36% fat) for 10 weeks had significantly increased their body weight compared to control animals (Kheder *et al.*, 2017).

7.3 How do fatty acids affect melanoma cell growth and macrophage function *in vitro*?

The role of tumour microenvironment (TME) in determining cancer development, progression and treatment outcomes has become increasingly evident in recent years. The TME is composed of non-malignant cellular and non-cellular components of the tumour niche, including, but not limited to, immune/inflammatory cells, blood cells, endothelial cells, fat cells and secreted proteins (Valkenburg *et al.*, 2018). Macrophages are a heterogeneous cell population and are frequently the most abundant immune cells in the tumour milieu, characterized by their plasticity and flexibility in representing a wide spectrum of activation states, ranging from the anti- tumour pro-inflammatory/classically activated (M1 macrophages) to tumour promoting, anti-inflammatory/alternatively activated (M2 macrophages). Recruitment of monocyte-macrophage lineage cells to the tumour microenvironment (TME), is one of the hallmark features of cancer-related inflammation. The tumour milieu contain tumour infiltrating myeloid cells which are rich in cancer cells and stromal components, and promote functions such as the enhancement of tumour growth, immunosuppression, angiogenesis, and cancer cell dissemination (Szebeni *et al.*, 2017). *In vitro*, animals and human studies found that fatty acids (FAs) have a significant effect on immune function. Several dietary studies found that NEFAs (non-esterified FAs; 'free FAs') and

triacylglycerol (TG) modulate several leucocyte functions such as proliferation, activation by antigens, cytokine release and cell death (de Jong *et al.*, 2014). Emerging evidence demonstrated that FAs modulate macrophage functions (Martins de Lima *et al.*, 2006). We identified that murine macrophages co-cultured with FAs had significant changes in their immunogenic activity, including an increase in production of pro-inflammatory cytokines TNF-α, CCL2, iONS and migration ability. Published data found that macrophages exposed to high levels of fatty acids during obesity activates inflammatory signalling pathways, promoting inflammation and metabolic dysfunction (Huynh *et al.*, 2018). Furthermore, fatty acid acts as DAMPs which activate TLR-4 and induce an inflammatory response with increased recruitment of macrophages (Kolb *et al.*, 2016). Interestingly, a potential paradox here is that obesity is associated with accumulation of M1-like polarized macrophages which are classically considered as anti-tumourgenic. Accumulating data suggested that tumours educate infiltrating cells when they enter the tumour microenvironment, so macrophages change their polarization to be a more tumour-promoting M2-like phenotype based on signalling that comes from the tumour (Martinez *et al.*, 2017). The results presented in this thesis found that conditioned medium of melanoma tumour B16-F10 enhanced macrophages expression of Arginase-1 mRNA and less TNF-α, suggesting that tumour cells may skew polarization of macrophages toward a tumour promoting M2 phenotype.

On the other, excess release of FAs from adipocytes during obesity, provides energy for rapid tumour growth and are considered building blocks for the synthesis of structural and signalling lipids to support the increase in cancer cell proliferation (Nieman *et al.*, 2011). Importantly, melanoma cells which are found in subcutaneous adipose tissue have been found to have high levels of palmitic acid levels. The increase in palmitic acids could be attributed to a high rate of *de novo* lipogenesis. *In vitro* studies showed that melanoma incorporated exogenous palmitic acid into structural and signalling lipids, suggesting that exogenous fatty acids, like palmitic acid may play an important role in melanoma growth and progression (Kwan *et al.*, 2014). This thesis showed evidence that FAs and HFD serum significantly enhanced tumour cells (B16-F10) proliferation, migration and increased cytoplasmic lipid inclusion *in vitro.* Lipidomic studies showed that lipid transferred from adipocytes were not only important for cancer growth but also suggest that tumorigenesis involves constant cross talk between cancer cells and neighbouring stromal cells such as adipocytes (Gazi *et al.*, 2007).

It can be conclude, that in *vitro* data presented in thesis not only implicates the pathological effects of FAs on macrophages and cancer cells in the tumour microenvironment (TME), but also suggests that reducing an exogenous supply of FAs to melanoma cells may be a potential therapeutic strategy that may help to inhibit melanoma cell proliferation and migration.

7.4 How could HFD induced obesity modulate tumour growth?

7.4.1 HFD induced systemic chronic low-grade inflammation with permanent increased in oxidative stress

It is generally accepted that dietary components are recognized as important modulators of inflammation, and healthy/unhealthy diets have been associated with reduced/increased different types of cancer, respectively (Donninelli *et al.*, 2017). Epidemiological data indicates that more than 25% of all cancers have a chronic inflammatory component and it is estimated that 15% of cancer related mortality is associated with inflammation (Ramos-Nino, 2013).

Chronic low-grade state of inflammation is the main characteristic of obesity, and it is recognized as a state that favours tumour initiation and development, largely through the infiltration of immune cells, secretion of pro-inflammatory mediators, excess fatty acids release and oxidative stress (OS), triggering a systemic acute-phase response (Mentoor *et al.*, 2018). Furthermore, an increase in oxidative stress levels leads to the cellular structural damage which promotes the development of obesity-related complications (Marseglia *et al.*, 2014). Obesity with hyperlipidemia can lead to the development of metabolic syndrome and is strongly related with oxidative stress on cellular level. Decreased circulating levels of high-density lipoprotein (HDL), increased triglyceride (TG) values and elevated plasma levels of very low density lipoprotein (VLDL) enhance the generation of reactive oxygen species (ROS) in the endothelium. Over generation of ROS can directly damage proteins or DNA and modulate intracellular signalling pathways like mitogen activated protein kinases, causing irreversible oxidative damage, contributing to carcinogenesis (Ceriello *et al.*, 2002; Sengenes *et al.*, 2007).

Activation of metabolic signalling pathways such as NF-*κB* and c-Jun N-terminal kinase (JNK) induced by excess release of nutrients such as free fatty acids leads to induction

of a low-level of inflammatory cytokines (tumour-promoting cytokines) such as IL-6, TNFα, IL-1β and increases the production of nitrogen oxide (NO) and favours formation of ROS (Gregor and Hotamisligil, 2011). During inflammation, increased production of ROS leads to DNA damage through different mechanisms such as DNA deletions, modifications and frame shifts. This damage can affect genes linked to cell survival or cell proliferation like p53 and RAS respectively, enhancing tumour progression and development (Valko *et al.*, 2004). Elevated systemic circulation and portal circulation of FFAs during obesity also lead to up-regulation of the expression of TLRs in circulating macrophages, enhancing macrophages activation to a M1 phenotype, which produces pro-inflammatory cytokines and chemokines (Duan *et al.*, 2018). Furthermore, macrophages have been shown to increase their infiltration into adipose tissue of obese individuals and is directly associated with overly stressed or necrotic adipocytes, leading to formation of crown-like structures. The number of these crown-like structures is positively correlated with the degree of inflammation within the adipose tissue, and is associated with activation of transcription factor nuclear factor κ B (NF-κB) (Brown and Simpson, 2012). This transcription factor plays an important role in inflammation and has emerged as an important endogenous tumour promote (Mantovani *et al.*, 2008). The data presented in this thesis provides further evidence about the role of a HFD as a pro- Inflammatory activator as we found significant up-regulation in circulating IL-6, TNF-α, and CCL2 (MCP-1) due to chronic consumption of dietary rich fat.

Recently ingestion of a high-fat diet has been shown to alter the gut microbiota, suggesting that changes in the composition of the gut microbiota and epithelial functions may play an important role in obesity related-inflammation (de La Serre *et al.*, 2010). Animal experiments and human trials showed a significant alteration in the composition of the gut microbiota of obese patients compared with lean controls (Ley *et al.*, 2005, Ley *et al.*, 2006). In mice, these modifications can be induced by consuming a diet rich in fat (Turnbaugh *et al.*, 2008; Cani *et al.*, 2008).

As inflammation contributes considerably to the development of many diseases with an inflammatory component including cancer, it is of fundamental importance to select diets that can modulate inflammatory pathways and therefore, lead to regulate and target immune systems in defined tumour microenvironments.

7.4.2 The indirect impact of a high fat diet on $CD4^+CD25^+FOXP3^+$ populations

Different expression profiles of genes important in immunity and altered immune cell function is usually accompanied by an excess of body fat in obese subjects (Gomez-Ambrosi *et al.*, 2004). During the onset of diet-induced obesity, adipose tissues have been shown to display a dynamic infiltration by innate and adaptive cells (Duffaut *et al.*, 2009). Although there are several lymphocyte cell types that are linked to obesity and cancer, $CD4^+$ $CD25^+$ regulatory T cells (T- regs) expressing the transcription factor FOXP3 have been shown to play an important role in obesity and cancer related inflammation (Takeuchi and Nishikawa, 2016). Despite the numerous studies investigating the role of T-regs in autoimmune diseases and cancer, the role of T-regs in diet-induced experimental obesity has only been recently identified. Published data found that T-regs play an important role in obesity-induced inflammation (Chao and Savage, 2018). The investigators found a marked increase in pro-inflammatory T cells such as $CD4^+$ and $CD8^+$ T cells and decrease in anti- inflammatory T cells like T-regs in visceral adipose tissue (VAT) of obese mice (Morin *et al.*, 2017).

There was also a reduction in visceral adipose T-regs and an increase in splenic T-regs due to HFD-induced obesity (Deiuliis *et al.*, 2011). These studies suggest that regulatory T cells at non-lymphoid sites mediate functions that are independent of immune suppression action. Additionally, adipose-resident T-regs cells have unique characteristics which differ from lymphoid organ T-regs cells, including responses to various environmental challenges to modulate immune responses under normal and pathogenic metabolic conditions (Zeng *et al.*, 2018). Cellular function and activity are controlled mainly by intracellular metabolism; recent studies demonstrated that metabolism and function of T-regs cells are significantly by local environmental conditions and the availability of certain metabolites (Galgani *et al.*, 2016). Similarly to conventional $CD4^+$ cells, regulatory T cells have a high degree of plasticity which associates with various transcriptional programs that in turn are influenced by cellular metabolism. However, T-regs cells differ from $CD4^+$ cells in their utilizing of fatty acids, glycolysis and fatty acid oxidation (FAO), in keeping with the environmental conditions that favour maintenance and expansion of T-regs (Procaccini *et al.*, 2016). Kim *et al.* (2015) who found that specific components in a commonly consumed diet can modulate T-regs cell properties and interaction with other cells within the tumour

microenvironment. In mouse models of non-alcoholic fatty liver disease, the level of hepatic T-regs ($CD4^+CD25^+FOXP3^+$) was decreased in mice consumed a HFD compared to mice fed on CD. However, splenic T-regs levels in mice fed a HFD remained unchanged (Ma *et al.*, 2007).

Due to a relative short history in which T-regs have been studied, investigations of the direct effects of various dietary components on T-regs actions are limited. Therefore, further studies are required to dissect the detailed mechanisms by which dietary components could influence T-regs function, differentiation and interaction with other cells. The tumour microenvironment is highly enriched in $CD4^+CD25^+FOXP3^+$ cells and their presence is associated with poor survival in solid tumours such as ovarian, breast, colorectal, lung, pancreatic cancer and melanoma (Facciabene *et al.*, 2012). Animal models showed that removal of $CD4^+CD25^+$ T-regs cells boost anti-tumour immune responses and the reduction in intra-tumoural T-regs induced tumour regression (Lutsiak *et al.*, 2008). Flow cytometry analysis demonstrated significant increases in $CD4^+CD25^+FOXP3^+$ from CD4 T cells in the spleen after consuming a fatty diet. Importantly, the increase in the frequency of $CD4^+CD25^+FOXp3^+$ cells in all animals mirrored the increase in tumour weight and size. In melanoma mouse models, the expression of Foxp3 enhances cancer progression by modifying the immune system at the local and peripheral level, skewing the milieu toward an immunosuppressive profile (Franco-Molina *et al.*, 2016).

This project utilizes available single cell suspensions from spleen to investigate for T-regs due to the importance of these immunosuppressive cells. T-regs cells were investigated in spleen but not in tumour due to the different obstacles such as low number of cells, which was insufficient to perform flow cytometry analysis, purity from the primary tumour and all tumours had to be processed within a few hours after harvesting to avoid degradation of tissue and alteration of antigen. It is important to note the difficulties that arose from carrying out a large scale of work when tissues from experimental mice were harvested once tumours reached to the endpoint (1.2 cm^3 in diameter). Time was restricted, as mice had to be weighted and dissected, measured of tumour weight, size and prepared of cell suspension from spleen. The depletion of regulatory T cells and reduction in their immune suppressive activity are two promising strategies that could work together or alone to improve anti- tumour therapies.Combinational immunotherapy which includes inhibition of suppressive

mechanisms, supplementing active immune elements and suppressing tumour growth and angiogenesis should be taken into consideration in the development of novel cancer treatments. Additionally, furthering our understanding of the TME and metabolic dysregulation will be beneficial to attain a comprehensive view of cancer biology and the improvement of cancer immunotherapy.

7.4.3 A high fat diet altered cytokine profile

Augmented adipose tissue in obesity produces increased amount of FAs, triglycerides, leptin and inflammatory cytokines compared to normal-weight individuals (Braun *et al.*, 2011). The ability of adipose tissue to generate factors that increase cell susceptibility to cancer initiation or progression has been supported by a variety of studies on different types of cancer (Cozzo *et al.*, 2011). During obesity, adipocytes profoundly alter the secretory profile of adipokines leading to development of oxidative stress and a proliferative microenvironment (Deng and Scherer, 2010). Leptin and Adiponectin are the major adipokines associated with obesity and their role in cancer cell growth and proliferation has been extensively explored in different studies (Ando and Catalano, 2011; Dutta *et al.*, 2012). However, the involvement of these adipokines in melanoma is not well understood. Beside its role in regulating energy balance, leptin plays an additional role as a pro- inflammatory "adipokine" with a wide range of activities including cytokine induction, cellular immunity, and inflammation (Mancuso, 2016). Murphy *et al.* (2018), who found that increases in leptin levels during diet-induced obesity reduce the efficacy of anti-tumour immunotherapy. Furthermore, exposure of ovarian cancer cells to leptin enhances cell migration and invasion due to the activation of the JAK/STAT3 pathway, PI3K/AKT signalling pathways downstream of the leptin receptor (Ob-Rs), which is associated with worse survival in overweight patients (Kumar *et al.*, 2017). The data presented in recent studies demonstrated that Leptin levels were significantly higher in obese mice sera bearing bigger tumours compared to lean animals.

Conversely, chemokine-receptor system can be altered dramatically in neoplastic tissue and chemokines induce direct effects on stromal and tumour cells in addition to their roles in coordinating leukocyte recruitment (Coussens and Werb, 2002). Accumulating evidence suggest that chemokines play an important roles in the tumour microenvironment and the migration of cancer cells are specifically regulated by the

expression of chemokines and their receptors (Vilgelm and Richmond, 2019). Our *in vitro* wound healing assay indicated a significant increase in B16 migration after treatment with HFD derived serum compared to CD serum. In cutaneous melanoma, expression of specific chemokine receptors facilitate melanoma cell migration into other organs to form secondary lesions preferentially in draining lymph nodes, lung, liver and the gut (Jacquelot *et al.*, 2018). By using proteomic analysis, the data presented in this study indicate significant alteration in cytokine secretion (IL-1 alpha, CXCL5, RegG3, RBP4, Osteoprotegerin and Pentraxin-3) in mice sera fed on a HFD compared to those fed on a CD. Sokol and Luster (2015), found secretion of CXCL5 by tumour cells and other immune cells induced recruitment of MDSCs and T-regs into the TME. Additionally, our data also showed that proteins involved in tumourgenesis, inflammation, obesity, diabetes, proliferation, invasion and migration were significantly altered after consumption of a high fat diet.

It can be concluded that the investigation using an *LDLR*$^{-/-}$ melanoma mouse model where the animals were fed a HFD has shown that obesity significantly enhances melanoma tumour growth and has immune modulatory effects on the tumour microenvironment (TME). This effect is mediated by different mechanisms which are difficult to dissect in a complex *in vivo* model, but which involve increased oxidative stress that promotes a systemic low-grade inflammatory response, elevated local and systemic production of pro-inflammatory adipokines which lead to production of ROS and dysregulation in chemokine secretion and infiltration of immune cells. All in all, the findings support the hypothesis of this study (**Figure 7-1**).

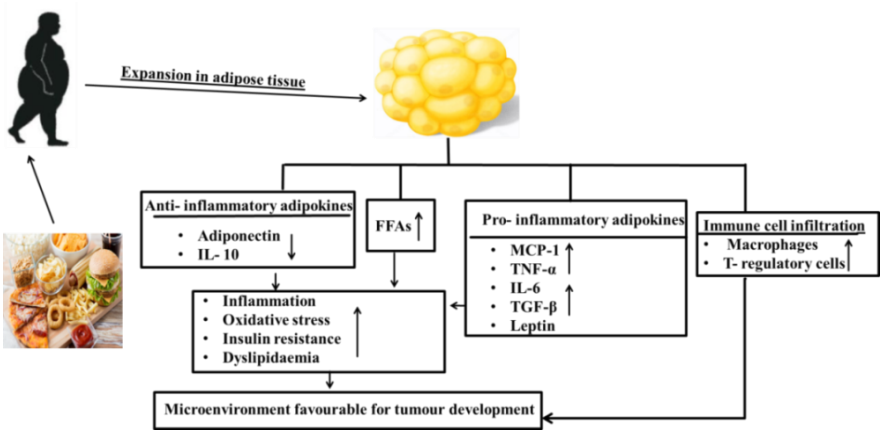


Figure 7-1: Roles of High Fat Diet- induced obesity in tumour growth.
Expansion of adipose tissue leads to elevate FAs, pro-inflammatory cytokines and reduces anti-inflammatory cytokines which provides an environment favouring tumour progression and development.

7.5 Does VitD3 influence B16-F10 melanoma cell characteristic *in vitro?*

VitD3 is a multifunctional precursor of the steroidal hormone calcitriol (1α,25-dihydroxyvitamin D3, 1,25(OH)2D3). Accumulating evidence found that vitamin D3 induces anti-proliferative effects on various cancer cell lines including those from prostate, breast, colon and gastric sites via mechanisms such as differentiation, apoptosis and growth arrest (Fleet *et al.*, 2012). Furthermore, the same investigators found that VitD3 induces a decrease in the growth rate of melanoma cells treated in a dose-dependent manner. Additionally, Spath *et al.* (2017) who found VitD3 impaired melanoma cell line proliferation and triggers differentiation after 72 hours. However, prolonging the time of exposure to 9 days leads to cell-cycle arrest. The results presented in this thesis demonstrated significant reduction in cell proliferation of B16 at high doses (10 µM) compared to low doses (1µM) which support the notion that the effect of Vitamin D3 is dose dependent.

Previous laboratory-based work (PhD thesis Khader 2017) showed that co –culture of murine macrophages (J774) with 10µM of VitD3 for 72 hours attenuated intracellular fat droplet accumulation in treated cells. Furthermore, Vitamin D3 at 100 nmol/L concentrations showed a decrease in intracellular fat accumulation in adipose cysts after 24 hours (Chang and Kim, 2016). Currently, the study found that 10µM of exogenous VitD3 added to HFD derived serum significantly decreased lipid inclusion in B16-F10 after 72 hours as indicated by Oil red O staining. Interestingly, VitD3 in HFD derived serum significantly enhanced cell proliferation of B16-F10 compared to cells treated with HFD derived serum which indicates that B16 melanoma cancer cells may locally dysregulate VitD3 metabolism. However, no significant differences were found in the migration ability between tested groups. It is important to mention that to our knowledge no previous study has investigated the effect of serum derived from mice fed on a HFD supplemented with VitD3 on any cancer cell line including melanoma cells (B16-F10). An anti-inflammatory role of vitamin D3 within the TME was highlighted. The present study investigated the anti-inflammatory role of vitamin D on melanoma cells. The results also showed the inability of exogenous VitD3 (10µM) added to HFD derived serum to impair the anti-inflammatory action of IL-6 on B16-F10 proliferation.

7.6 What influence does VitD3 have on tumour growth, activity and the composition of the immune microenvironment on an obese background?

To address, whether vitamin D3 plays an important role in obesity and tumour growth. *LDLR*$^{-/-}$ mice fed on either HFD or a HFD supplemented with VitD3 was used to investigate the role of a VitD3 on melanoma tumour growth in a mouse model of obesity. The direct /indirect association between VitD3 and overweight/obesity has been suggested by several lines of evidence (Merino *et al.*, 2017). Published studies reported that obese individuals had the lowest VitD3 compared to lean ones (Zakharova *et al.*, 2019). However, the mechanism underlying this association remains controversial. Wang *et al*. (2017), who investigated the effect of a VitD3 supplemented HFD using female C57BL/6J mice fed on a HFD (45% fat) for 10-weeks. Their study reported that supplemental vitamin D3 had no effect on preventing HFD-induced obesity. In the current study, measurement of body weight indicates that after 12-week consumption of a HFD and VitD3 or a HFD alone in *LDLR*$^{-/-}$ tumour brings mice, there were no significant differences in weight gain between the groups. Secondly, we hypothesised that the anti-inflammatory action of Vitamin D3 could modulate tumour growth in obese mice fed on a diet rich in fat. Many, although not all, studies suggested an association between vitamin D3 levels and cancer risk (Zakharova *et al.*, 2019). A meta-analysis study found that melanoma patients had insufficient levels of VitD3 than healthy controls. Furthermore, Vitamin D3 levels are known to be lower in obese individuals and several studies have observed that increased BMI is associated with an increased risk of developing melanoma (Shanmugalingam *et al.*, 2014).

In spite of accumulating studies supporting the notion that vitamin D3 has cancer prevention action and its deficiency is associated with increase cancer risk, our results found that mice fed on (10 IU/g) VitD3 supplemented HFD had significantly larger tumours compared to mice fed on a HFD alone. Accumulating data suggests that malignant cells utilize several mechanisms that decrease cellular calcitriol (active form of VitD3) levels, as well as diminishing its action to protect themselves from the anti-tumorigenic effects of VitD3 (Jeon and Shin, 2018). Despite increasing in tumour weight and size of *LDLR*$^{-/-}$ tumour bearing mice fed a HFD plus VitD3, flow cytometry of splenocytes showed a significant reduction in the T-regs population from total $CD4^{+}$

($CD4^+$ $CD25^+$ $FOXP3^+$) cells compared to those fed on a HFD. This finding may be explained by the infiltration of T-regs into tumour site (although this was not examined). Therefore, further analysis is required to investigate whether these cells have trafficked to the tumour mass. Enhanced cell proliferation of T-regs ($CD4^+CD25^+FOXP3^+$) cells within the TME leads to intra-tumoral accumulation of T-regs and it is associated with poor prognosis and a reduced survival rate (Tang *et al.*, 2014). The accumulation of T-regs may be due to several factors including increased proliferation, decreased apoptosis, and altered expression of chemokines and chemokine receptors that mediate trafficking (Ha, 2009). Inflammation plays an important role in tumorigenesis, epidemiological and clinical observations VitD3 exerts anti-inflammatory effects by inhibiting the secretion and action of pro-inflammatory mediators such as cytokines (Wu *et al.*, 2018). Quantitative measurement of circulating adipokines revealed no significant differences in the levels of IL-6, Leptin, TNF- α, CCL2 and IL-10 in the sera of $LDLR^{-/-}$ tumour bearing mice fed for 12-weeks with a HFD and a HFD with VitD3. A published study reported that VitD3 can regulate the interaction between infiltrating immune and cancer cells in TME which inhibits the secretion of pro-inflammatory cytokines. Furthermore, the anti-inflammatory action of VitD3 can be mediated by inhibiting the nuclear factor kappa B (NF *κB*) signalling pathway (Jeon and Shin, 2018).

Conclusion

In spite of large progress that has been made in understanding the complexity of VitD3 biology in neoplastic cells, its influence on the nutrient utilization of tumours remain unclear. Furthermore, VitD3 may induce reprogramming of the tumour stroma by modulating microenvironment conditions that are known to promote oncogenesis such as inflammation. Additionally, a better understanding of cellular signalling pathways involved in metabolic dysregulation of VitD3 in cancer cells could provide a promising strategy for using vitamin D3 in cancer therapy.

7.7 What are the implications of this study finding for therapy?

While genetic factors influences the risk of various types of cancer, most of the variation in cancer occurrence across populations and among individuals is due to lifestyle and environmental factors (Wilson *et al.*, 2002). Demonstration of a link between diets rich in fat and various types of cancer has encouraged researchers to find a strategy to reduce HFD-driven diseases and cancer (Prieto-Hontoria *et al.*, 2011, Rock *et al.*, 2012). Data from experimental and epidemiological studies indicate that expansion of adipose tissue due to consuming rich caloric diets and minimal physical activity increases the risk of developing multiples types of cancer (Kampman *et al.*, 2012). This project showed that mice fed a diet rich in fat showed a significant increase in weight gain, developed heavier and bigger tumours compared to their counterparts fed on a low fat diet.

Despite advances in anti-obesity drugs, diet-controlled interventions remain the preferred line of therapy for effective management of obesity. It has been suggested that weight-loss interventions could be important in the prevention of obesity-promoted cancer progression by reducing the amount of adipose tissue, reducing serum levels of adipokines that affect tumour promoting molecules and signalling pathways (Malvi *et al.*, 2015). Furthermore, dietary approaches have also shown efficiency in reducing obesity-related inflammation (Doerstling *et al.*, 2017). Published studies reported a reduction in pro-inflammatory cytokines levels and C-reactive protein in the sera of overweight and obese patients after undergoing significant (>10%) weight loss (Bastard *et al.*, 2000; Jung *et al.*, 2008). Dietary restrictions have been shown to reduce the expression of inflammatory transcription factors in cancer cells, including *NF-κB* and signalling pathways involved in regulating inflammation, proliferation, and lipid homeostasis (Peters *et al.*, 2012; Staudt, 2010). An increasing body of evidence demonstrated that fasting can sensitise tumours to chemotherapy and radiotherapy, suggesting that a combined action of dietary restriction and anticancer therapy may be sufficient to overwhelm cancer cell development (Vernieri *et al.*, 2016). Experimental animals using murine models show conflicting results on the benefits of losing weight relevant to inflammation and cancer. Those studies utilizing robust calorie restriction demonstrate a remarkable reduction in inflammatory mediators and cancer development (Bhardwaj *et al.*, 2013), compared with studies that only have modest dietary weight loss strategies (Rossi *et al.*, 2016). Calorie restriction -mediated beneficial effects on

cancer, with reduction in the production of growth factors, modulation of endogenous antioxidant systems, (which decrease oxidative stress and free radical-induced DNA damage) and increase in Adiponectin secretion (O'Flanagan *et al.,* 2017). Elevated leptin levels have been reported in various types of cancer including melanoma (Malvi *et al.*, 2018) In contrast, Adiponectin exerts multiple key functions via its anti-metabolic syndrome, anti-inflammatory properties and anti-tumorigenesis activity (Karnati *et al.*, 2017). Several published studies reported an inverse association between adiponectin concentrations and the risk of various types of cancer such as renal, breast, endometrial, gastric and prostate cancer (Barb *et al.*, 2006; Gavrila *et al.*, 2003; Li *et al.*, 2010). Thus, antagonizing the actions of Leptin by Adiponectin could be a promising therapeutic approach.

In summary, considering lifestyle factors of metabolic diseases is an integral component in the management of various aspects of tumorigenesis and tumour progression. Dietary interventions aimed to lowering the circulating levels of FFAs, reducing adipokine signalling mediators and limiting inflammation would be an appropriate approach to minimise the risk of obesity-promoted cancer progression and development.

7.8 Limitations

Importantly, Due to the ethical considerations of our licence we were restricted with tumour size growth to 1.2 cm^3, which did not allow us to investigate tumour progression further. This study might appear limited by the number of mice that used, however, we analysed both females and males in their respective litter groups.

It was necessary in the designing of the experiments to probe mice from the same litters for their reaction to the diet and treatments. The study found differences in ability to obtain weight gain between litters, therefore, littermates were analysed in this study to ensure more uniformity in diet induced metabolic reaction. All mice were kept in the same barrier facilities and they were exposed to the same handling and procedures. One of limitation is that *in vivo* experiments analysed only T-regs percentage in the spleen; however, other cell populations are interesting to look at. Furthermore, the study only sampled spleen it would be interesting to sample tumour and bonne marrow (BM) with respect to phenotyping of cell populations. Another limitation regarding Vitamin D3 is that the T-regs population was not investigated in naïve mice. Furthermore, obese mice

with vitamin D3 have larger tumours compared to those without vitD3, so it is now of interest to investigate the effect of VitD3 in lean mice fed on control diet (CD). Additionally, Vitamin D3 levels were not determined as part of this study, however, a previous study had established that the VitD3 enriched diet used in the present study led to 3.5 fold increase of circulating vitamin D3 in *LDLR*$^{-/-}$ in mice.

The conclusions presented are derived from mice fed a high fat diet for duration of up to 12-weeks. This limit was imposed by welfare consideration so that the experimental mice would remain on a moderate protocol. In human, however, unhealthy diets are consumed for much longer, so conclusions need to be extrapolated with caution.

7.9 Future Directions

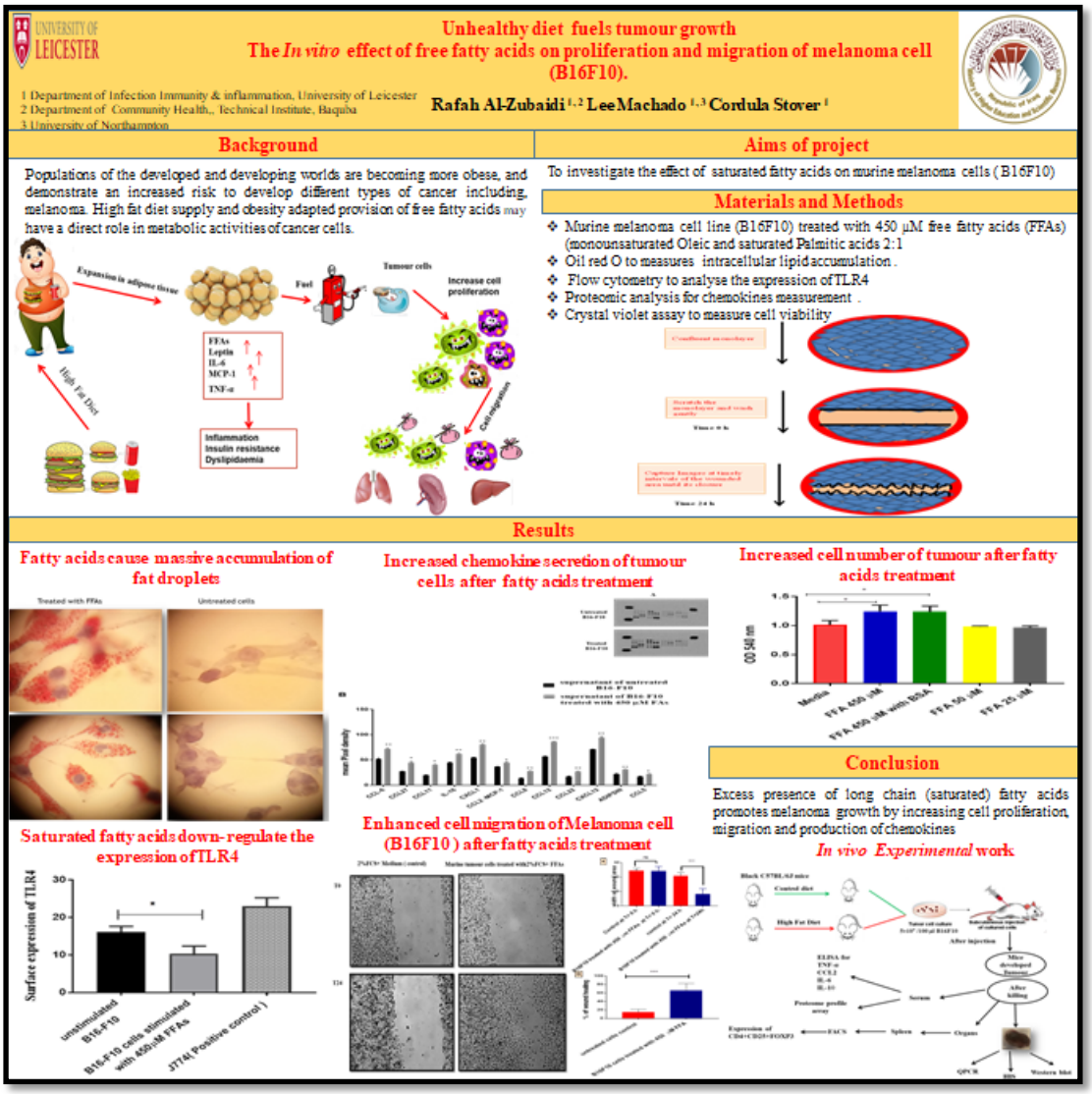
Obesity, which is reaching pandemic levels, is also considered a negative prognostic factor for multiple pathologic states, including cancer. Chronic low-grade systemic innate immune activation is a main feature of obesity and accelerates cancer growth, promotes cancer recurrence and worsens the chances of survival in cancer patients. During the last decade the use of cancer immunotherapy has gained momentum, achieving notable success in various malignancies (Murphy and Longo, 2019). Among all immunotherapies, the use of immune checkpoint blockade inhibitors has been revolutionary for melanoma therapy where responses to treatment have been poor. However, the effects of checkpoint blockade inhibitors in obese patients, who present a heightened inflammatory state, remain to be evaluated. Therefore, further studies are needed to investigate the effect of obesity-related chronic inflammation in response to melanoma immunotherapy. In addition, recent evidence has underlined a possible association between the alteration in gut microbiota (microbiome) in obese patients and certain types of cancers (Rogers *et al.*, 2010). In melanoma, the microbiome modulates the efficacy of immunotherapies (Sivan *et al.*, 2015; Vetizou *et al.*, 2015). Furthermore, previous publications also shown that Vitamin D3 can affect the gut microbiome (Tabatabaeizadeh *et al.*, 2018), and so the interaction between diet, the microbiome and response to immunotherapy should be investigated in obese melanoma patients receiving immunotherapies in the future.

Chapter 8

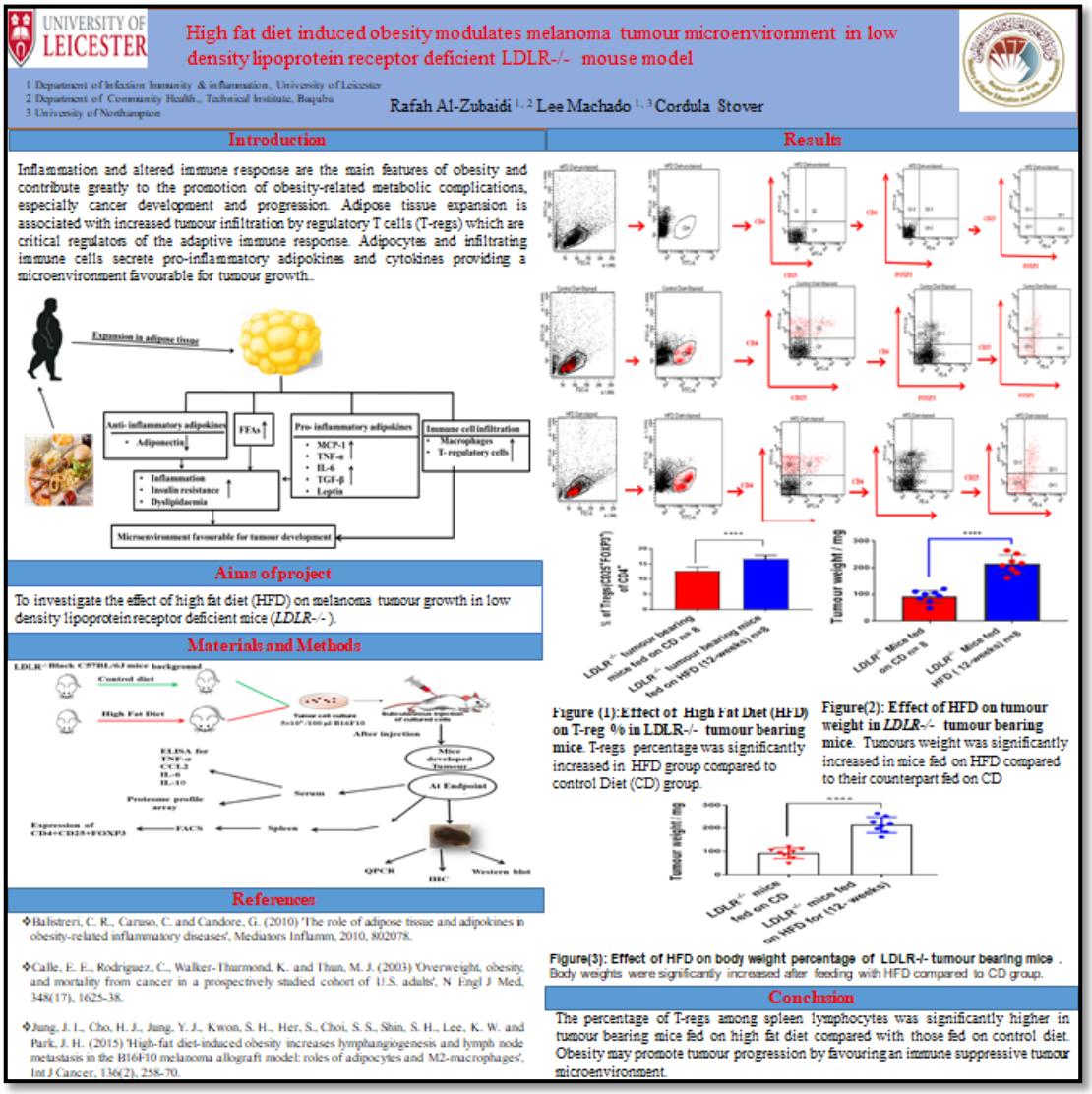
Appendices

Appendices

Appendix 1: Royal Society of Biology Symposium, university of DE Montfort, UK, May 2018



Appendix 2: 14th Euro Obesity and Endocrinology Congress September 13-14, 2018, London, UK (winner of Best poster presentation).



UNIVERSITY OF LEICESTER

High fat diet induced obesity modulates melanoma tumour microenvironment in low density lipoprotein receptor deficient LDLR-/- mouse model

1 Department of Infection Immunity & inflammation, University of Leicester
2 Department of Community Health,, Technical Institute, Baquba
3 University of Northampton

Rafah Al-Zubaidi [1, 2] Lee Machado [1, 3] Cordula Stover

Introduction

Inflammation and altered immune response are the main features of obesity and contribute greatly to the promotion of obesity-related metabolic complications, especially cancer development and progression. Adipose tissue expansion is associated with increased tumour infiltration by regulatory T cells (T-regs) which are critical regulators of the adaptive immune response. Adipocytes and infiltrating immune cells secrete pro-inflammatory adipokines and cytokines providing a microenvironment favourable for tumour growth..

Aims of project

To investigate the effect of high fat diet (HFD) on melanoma tumour growth in low density lipoprotein receptor deficient mice (*LDLR-/-*).

Materials and Methods

References

- Balistreri, C. R., Caruso, C. and Candore, G. (2010) 'The role of adipose tissue and adipokines in obesity-related inflammatory diseases', Mediators Inflamm, 2010, 802078.
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Results

Figure (1):Effect of High Fat Diet (HFD) on T-reg % in LDLR-/- tumour bearing mice. T-regs percentage was significantly increased in HFD group compared to control Diet (CD) group.

Figure(2): Effect of HFD on tumour weight in *LDLR-/-* tumour bearing mice. Tumours weight was significantly increased in mice fed on HFD compared to their counterpart fed on CD

Figure(3): Effect of HFD on body weight percentage of LDLR-/- tumour bearing mice . Body weights were significantly increased after feeding with HFD compared to CD group.

Conclusion

The percentage of T-regs among spleen lymphocytes was significantly higher in tumour bearing mice fed on high fat diet compared with those fed on control diet. Obesity may promote tumour progression by favouring an immune suppressive tumour microenvironment.

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17th October 2018

Dear Rafah,

I would like to take this opportunity to congratulate you on being awarded the prize for best poster presentation at the 14th Euro Obesity and Endocrinology Conference held on 13th to 14th September 2018. This is a great achievement and a credit to the department of which you should be very proud.

Yours sincerely

David Cousins

Professor David Cousins

Interim Head of Department

Cc: Dr Cordula Stover and Dr Lee Machado (Supervisors)

Athena SWAN Silver Award

Appendix 3: College of Life Sciences Poster Fair University of Leicester, Leicester, UK, March 2019.

UNIVERSITY OF LEICESTER

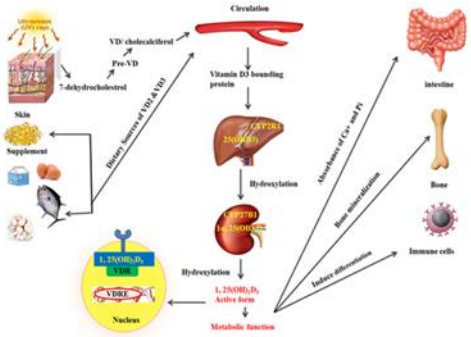
LDLR-/- mouse melanoma model cautions against consumption of Vitamin D.

Rafah Al-Zubaidi 1, 2 Lee Machado 1, 3 Cordula Stover

1 Department of Infection Immunity & inflammation, University of Leicester
2 Department of Community Health,, Technical Institute, Baquba
3 University of Northampton

Introduction

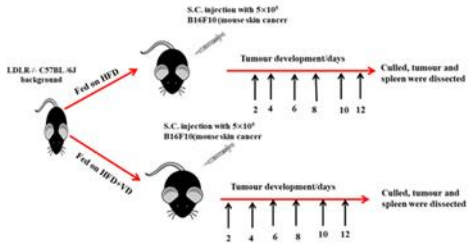
During the past decade, the non-classical actions of Vitamin D, such as pro-differentiation, immune function modulation, and anti-inflammation, have received much attention. The potential role of vitamin D analogs in combination with anticancer agents for the treatment of different types of cancer has recently been studied. The association between vitamin D status and the higher incidence of many forms of cancer has suggested that vitamin D may play a role in the etiology of these types of cancer. However, the mechanism behind this action is still controversial. Intracellular metabolism is central to cell activity and function, recent studies have shown that metabolism and function of Treg cells are influenced significantly by local environmental conditions and the availability of certain metabolites. The presence of specific metabolites in the microenvironment profoundly affects Treg differentiation, expression of FOXP3, and phenotype stabilization..



Aims of project

To investigate the effect of high fat diet (HFD) supplemented with Vitamin D on melanoma tumour growth in low density lipoprotein receptor deficient mice (LDLR-).

Materials and Methods



References

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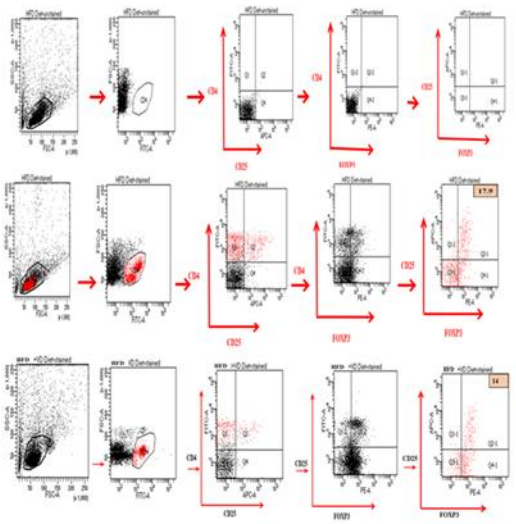
Results



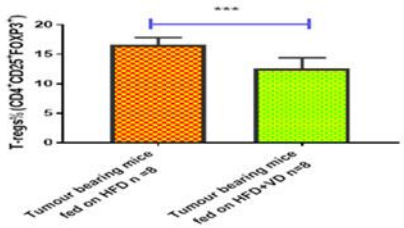


Figure (1):Effect of High Fat Diet (HFD) on T-reg % in LDLR$^{-/-}$ tumour bearing mice. T-regs percentage decreased in HFD+VD group compared to high fat diet group (HFD.

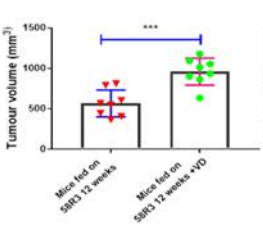


Figure (2): Effect of HFD+VD on tumour size in LDLR$^{-/-}$ tumour bearing mice. Tumours size was significantly increased in mice fed on HFD +VD compared to their counterpart fed on HFD

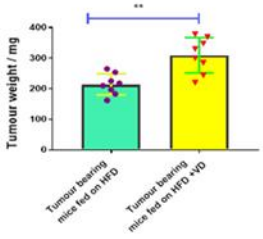


Figure (2): Effect of HFD+VD on tumour weight in LDLR$^{-/-}$ tumour bearing mice. Tumours weight was significantly increased in mice fed on HFD +VD compared to their counterpart fed on HFD

Conclusion

Vitamin D supplementation may enhance melanoma tumour progression in obese LDLR-/- mouse model due to its anti-inflammatory action.

Table 8-1: proteins that were assessed in the protein array

chemokines	**CD**	**HFD**	***P* values**
CCL11	15.94 ± 0.43	17.89 ± 0.74	0.1503 ns
CCL2/ MCP-1	36.69 ± 32.16	37.79 ± 35.22	0.9838 ns
CCL21/6Ckine	41.25 ± 0.35	43.03 ± 0.30	0.1506 ns
CCL22/MDC	8.934 ± 0.38	11.33 ± 0.40	0.0500 ns
CCL5	7.827 ± 0.35	9.037 ± 0.49	0.1817 ns
Chemerin/CCL12	37.98 ± 0.75	40.7 ± 0.46	0.0899 ns
Adhesion molecules	**CD**	**HFD**	***P values***
P-selection	51.05 ± 0.39	56.06 ± 0.36	**0.0110***
CD54/ICAM-1	45.29 ± 0.54	49.05 ± 0.22	**0.0228***
VCAM-1/CD106	38.17 ± 0.67	44.24 ± 0.05	**0.0121***
E-Selectin	51.23 ± 0.57	56.92 ± 0.54	**0.0185***
Periostin	30.12 ± 0.23	32.35 ± 0.53	0.0604 ns
Cytokines	**CD**	**HFD**	***P* values**
IL-1 alpha	10.3 ± 0.42	18.14 ± 0.71	**0.0110***
IL-4	10.05 ± 0.3	12.97 ± 0.933	0.0968 ns
Fetuin A/AHSG	28.23 ± 0.46	30.97 ± 0.93	0.1174 ns
CXCL5	103.9 ± 0.67	112.1 ± .79	**0.0154***
C reactive protein	47.8 ± 0.66	51.1 ± 0.82	0.0884 ns
Reg3G	95.05 ± 0.28	100 ± 0.86	**0.0313***
Osteopontin	56 ± 0.61	58.03 ± 0.16	0.0845 ns
RBP4	17.21 ± 0.48	20.86 ± 0.53	**0.0368***
Osteoprotegerin	14.09 ± 0.28	16.71 ± 0.39	**0.0318***

CD257/ BAFF	56.96 ± 0.22	57.73 ± 1.12	0.5643 ns
Pentraxin-3	28.01 ± 0.11	30.29 ± 0.26	**0.0148***
Growth Factors and tumour angiogenesis	**CD**	**HFD**	***P* values**
Angiopoietin 1	57.04 ± 0.90	62.96 ± 0.44	0.0276
Angiopioetin-2	57.93 ± 0.71	61.03 ± 0.78	0.0995 ns
Angiopioetin-like3	48.94 ± 0.38	51.76 ± 0.69	0.0692 ns
M-CSF	14.71 ± 0.46	20.96 ± 0.59	**0.0141***
VEGF-A	52.06 ± 0.36	62.48 ± 1.08	**0.0117***
FGF acidic	33.1 ± 0.55	39.89 ± 0.57	**0.0133***
Gas 6/TAM	27.83 ± 0.27	29.17 ± 0.3	0.0803 ns
EGF	21.55 ± 12.62	23.84 ± 12.51	0.9092ns
DKK-1	15.31 ± 0.45	12.6 ± 0.41	**0.0466***
Insulin family proteins , receptors, and ligands	**CD**	**HD**	***P* values**
Flt-3 Ligand	15.96 ± 0.42	13.03 ± 0.94	0.1044
Resistin	53.29 ± 0.55	57.31 ± 0.49	**0.0321***
Leptin	32.38 ± 0.46	40.77 ± 0.72	**0.0106***
Adiponectin	73 ± 0.42	68.85 ± 0.21	**0.0122***
IGFBP-1	29.21 ± 0.11	34.95 ± 0.77	**0.0179***
IGFBP-2	60.89 ± 0.57	63.89 ± 0.57	0.0644 ns
IGFBP-3	54.83 ± 0.58	57.27 ± 0.63	0.1034 ns
IGFBP-5	27.73 ± 0.39	31.28 ± 0.69	**0.0463***
IGFBP-6	52.19 ± 0.23	53.77 ± 0.41	0.0776 ns
Enzymes	**CD**	**HD**	***P* values**

CD26/DPPIV	47.38 ± 0.41	43.96 ± 0.10	**0.0151***
Cystatin C	23.13 ± 0.52	26.04 ± 0.84	0.0985
Endostatin	57 ± 0.48	57.88 ± 0.72	0.4108
Chitinase 3-like 1	38.96 ± 0.32	42 ± 0.78	0.0692
Myeloperoxidase	30.13 ± 0.42	37.82 ± 0.68	**0.0106***
MMp-2	37.67 ± 0.51	41.2 ± 0.93	0.0791 ns
MMp-3	44.07 ± 0.9	47.52 ± 0.49	0.0780 ns
MMp-9	46.05 ± 0.89	51.21 ± 0.57	**0.0397***
Proprotein Convertase 9/	64.25 ± 0.65	74.43 ± 1.29	**0.0196***
Complement	**CD**	**HD**	***P* values**
C1-qR1/CD93	32.33 ± 0.59	33.86 ± 0.47	0.1806 ns
complement D	31.64 ± 0.62	34.47 ± 0.5	0.0707 ns
C5/C5a	21.77 ± 0.38	24.27 ± 0.65	0.0795 ns
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MMp-9	46.05 ± 0.89	51.21 ± 0.57	**0.0397***
Proprotein Convertase 9/	64.25 ± 0.65	74.43 ± 1.29	**0.0196***

Complement	CD	HD	*P* values
C1-qR1/CD93	32.33 ± 0.59	33.86 ± 0.47	0.1806 ns
complement D	31.64 ± 0.62	34.47 ± 0.5	0.0707 ns
C5/C5a	21.77 ± 0.38	24.27 ± 0.65	0.0795 ns

* The data are presented as means ±SEM (the experiment was repeated three times in triplicate each), 4 mice per group and statistical analysis was performed by Two-way ANOVA Sidak's multiple comparisons test. * ($p < 0.05$).

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