The role of the adipokine chemerin in prostate cancer

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Medicine. It has been composed by myself and has not been submitted in any previous application for any degree.

Abstract

Obesity is a major health problem worldwide and its effects on the cardiovascular system are well documented. It also leads to the development of metabolic disease such as insulin resistance and diabetes. There is evidence that obesity leads to an increased risk of developing numerous malignancies. Indeed, obese individuals diagnosed with malignancy tend to have poorer outcomes in terms of survival. A possible explanation for this is through the action of obesity-related cytokines (adipokines). These may play a role in either propagating, or perpetuating carcinogenesis and I explored the role of one particular adipokine: chemerin in prostate cancer. Prostate cancer cell lines (PC3 & LNCaP) were used for cell proliferation, migration, invasion and apoptosis assays. Western blot analysis and qRT-PCR techniques were used to evaluate the effects of chemerin on levels of key intracellular agents of carcinogenesis (bcl-2, p53, ERK and AKT) as well as novel, pro-cancerous genes such as anterior gradient 2 (AGR2). Serum samples were obtained from adult men with prostate disease to evaluate whether chemerin is associated with body parameters. Chemerin exerts positive effects on cellular proliferation and migration as well as inhibition of apoptosis in prostate cancer cells. These effects may be mediated by increased expression of the oncogene: bcl-2. Bcl2 expression was elevated in both cell lines after 24 hours stimulation with chemerin at increasing doses. Chemerin also appeared to cause activation of ERK and AKT pathways in prostate cancer cells as well as increased expression of the pro metastatic AGR2 gene at both the mRNA and protein level. An ELISA demonstrated chemerin behaving as an adipokine in adult men with prostate disease in keeping with previously published data. Chemerin certainly appears to play a role in prostate carcinogenesis, at least at the cellular level.

1 Introduction

1.1 The Prostate: basic anatomy, pathology and physiology

The prostate gland is an organ of the male reproductive tract, found deep in the male pelvis. It usually weighs about 11grams and its size is often compared to that of a walnut or horse chestnut. The prostate gland is surrounded by the symphisis pubis anteriorly, the rectum posteriorly, deep perineal fascia inferiorly, the urinary bladder superiorly and the levator ani muscles (part of the male pelvic floor) laterally. The prostate is enclosed by a thin but firm fibrous capsule or prostatic sheath and it is sheathed within the muscles of the pelvic floor. The prostate is connected to the symphisis pubis by the pubo-prostatic ligaments. The prostate surrounds the neck of the bladder and the first part of the male urethra (prostatic urethra) which is the widest part of the urethra. The urethra can become compressed here through a condition called benign prostatic hyperplasia (BPH – pathological diagnosis) or benign prostatic enlargement (BPE – clinical diagnosis). BPH leads to enlargement of the prostate gland and is a condition that is more common in older men. BPH can impede urine flow out of the bladder and can cause a variety of symptoms or even stop urinary flow completely. About two thirds of the prostate gland is composed of glandular tissue, the rest being composed of muscular tissue (smooth muscle). About 20-30 tubular-alveolar glands open into the prostatic urethra and they release prostatic fluid during ejaculation.

The prostate can be divided anatomically by **two** common classification methods:

Zones:

- Peripheral zone (70-80% of prostate cancers arise in this area)
- Central zone
- Transition zone
- Fibro-muscular zone

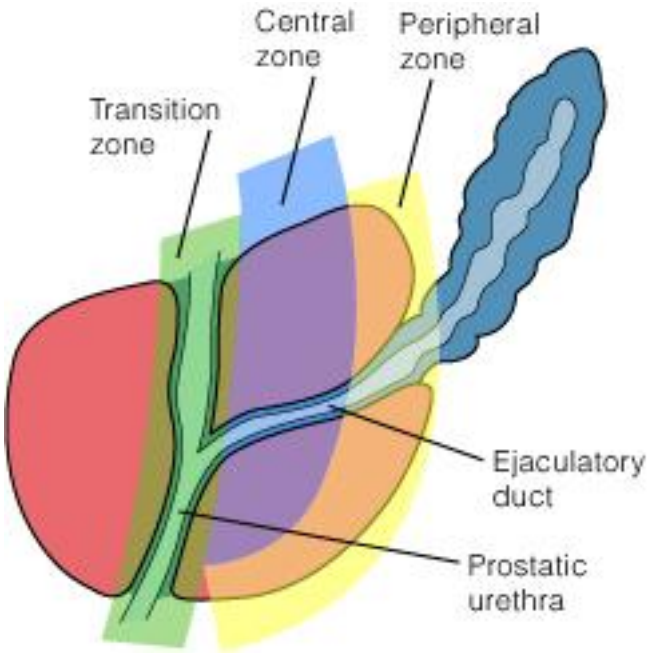

Figure 1-1: Diagram demonstrating zones of prostate (Image courtesy of www.pathologyoutlines.com).

Lobes:

- Anterior lobe
- Posterior lobe
- Lateral lobes
- Median (middle) lobe

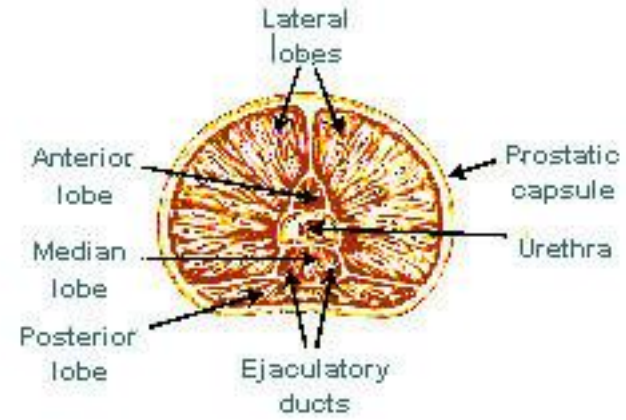

Figure 1-2: Diagram demonstrating lobes of prostate (Image courtesy of www.myprostate.com.au).

The main function of the prostate gland is the production of the slightly acidic, milky, nutrient-rich prostatic fluid which comprises between 20-30% of final seminal fluid volume. This fluid nourishes the sperm and contributes to the optimum pH in the female genital tract to assist successful fertilisation.

Contents of prostatic fluid:

- Citrate
- Fibrinolysin
- Hyaluronidase
- Acid phosphatase
- Prostate-specific Antigen (PSA)
- β-microseminoprotein
- Zinc

1.2 Prostate cancer: Epidemiology

Prostate cancer (PCa) is the most prevalent cancer in men in the UK, accounting for 25% of all male cancer diagnoses. PCa predominantly affects older men however 20% of cases occur in men younger than 65 and the incidence is higher in the Afro-Caribbean population. There were 34 335 men diagnosed with PCa in 2008 (Cancer Research UK (2008) www.cancerresearch.org.uk).Although the vast majority of PCa in men remains undetected, PCa is still a leading cause of mortality usually due to metastatic disease (1) and was, responsible for 9376 deaths in the year 2008 in the UK. The need to identify practical and reliable markers for aggressive PCa in order to accurately risk stratify patients is therefore greater than ever (2). The introduction of PSA testing and Trans-rectal Ultrasound (TRUS) prostate biopsy has in part lead to an increased incidence of PCa, particularly in the Western world. Currently, far fewer men present with advanced disease than was the case 30 years ago. PSA testing however leads to the potential over-diagnosis and subsequent over-treatment of men with prostate cancer. Men may suffer potential morbidity and even mortality from the treatment of a disease that may not be causing, or going to cause them any harm. There is evidence that over 80% of men aged 81-95 have histological evidence of prostate cancer but die from non-urological causes (3). Although the pathogenesis of PCa is multi-factorial it is hoped that by using selective bio-markers we will be able to identify those with more aggressive disease who are more likely to benefit from earlier treatment whilst hopefully identifying patients with prostate cancer who can be safely monitored and those who need no treatment at all.

1.3 Obesity and prostate cancer

Obesity is now a chronic disease in its own right and carries with it harmful implications not only on the cardiovascular system but on every organ system within the body. Obesity is a major health concern worldwide, especially in the United Kingdom (UK). Recent evidence suggests that the prevalence of abdominal obesity (possibly a more harmful form of obesity) in primary care in men in the UK is estimated to be as high as 39%, (4) compared to nearly twenty years ago when the prevalence of obesity was much lower in the UK at 9% (5). It is perhaps difficult to compare rates of obesity directly between these two cohorts as the earlier group measured obesity according to body mass index (BMI = weight (Kg)/height (m^2)) and the latter according to waist circumference (cm). Nevertheless this evidence suggests an alarming trend at an increase in obesity in recent years. The rise in obesity is multi-factorial but factors such as a western diet and a sedentary lifestyle are likely to be key players. The impact that this will have on the health of the general population remains to be seen but there is expected to be a rise in insulin resistance, diabetes mellitus (DM) and cardiovascular diseases such as coronary artery disease (CAD) and ischaemic heart disease (IHD). There is evidence that obesity leads to an increased risk of developing numerous cancers, possibly including prostate cancer, but more importantly an increased risk of prostate cancer-specific mortality. A large cohort of 404 576 American adult men were recruited for study and followed up was for a period of 16 years. Cause of death was noted according to the International Classification of Diseases, Ninth Revision (ICD-9). Men with a BMI, less than 18.5 were not recruited into the study and all participants were clear of malignancy at the time of recruitment (other than non-melanomatous skin cancer). Those without a recorded height or weight were excluded as it was not possible to calculate their BMI. A potential recognised limit of the study is that height and weight was self-reported through a questionnaire and included both their current weight and their weight one year earlier. This reporting bias could have led to potential inaccuracies in the later analyses. Those who had lost more than 4.5Kg of weight loss in the previous year were also excluded from analyses.

The Relative Risk of **death** from prostate cancer for each group according to their BMI is outlined in Table 1-1(6):

BMI (Kg/m^2)	Number of deaths from Prostate cancer	Death rate (standardised rate per 100 000)	Relative Risk (95%CI)
18.5 – 24.9	1,681	67.36	1.00
25.0 – 29.9	1,971	73.02	1.08 (1.01–1.15)
30.0 – 34.9	311	83.00	1.20 (1.06–1.36)
35.0 – 39.9	41	87.35	1.34 (0.98–1.83)
	4 004		**p < 0.001**

Table 1-1: Table demonstrating relationship between BMI and relative risk of death from PCa (Calle et al., 2003).

Relative Risk was adjusted for age, education, smoking status and number of cigarettes smoked, physical activity, alcohol use, marital status, race, aspirin use, fat and vegetable consumption. Although the change in relative risk is fairly minimal with regards to increasing BMI there is a definite trend towards an increasing risk of mortality from prostate cancer with increasing BMI. Due to the high prevalence of obesity, a small increase in the relative risk of mortality from prostate cancer due to an increased BMI is potentially significant. Andersson et al., published one of the largest studies to date, in terms of patient numbers, evaluating the role of obesity and prostate cancer in 1997 (7). 135 006 Swedish construction workers were recruited from 1971 and 1975 for a preventative health check-up which was carried out at regular intervals by a mixture of nurses and physicians. Data was recorded centrally on a computerized database. Approximately 200 parameters were recorded including marital status, smoking history, height and weight. A strength of this study is that height and weight were recorded in a standardised way and not self-reported as per the New England Journal of Medicine (NEJM) study and avoids the self-reporting bias that this entails. Men were followed for a an average of 18 years until the diagnosis of prostate cancer, migration, death or the cut-off date (Dec 1991), whichever came first. This

resulted in 2 369 009 person-years for the analyses of the incidence of prostate cancer and 2 377 960 person years for the analyses of mortality rate. Outcomes for prostate cancer incidence, emigration and mortality were found using the Swedish National Cancer Register (coded according to ICD-7), the Migration Register and the National Death Register respectively. The table below demonstrates the relationship between BMI, prostate cancer incidence and prostate cancer mortality (Table 1-2).

BMI (Kg/m^2)	Number of cases of Prostate cancer	Relative Risk (Incidence)	Number of deaths from Prostate cancer	Relative Risk (Mortality)
<22.1	290	1.0	74	1.0
22.1-24.1	499	1.09	155	1.36
24.2-26.2	676	1.10	202	1.33
>26.2	899	1.13	277	1.40
	2 364	**p = 0.10 (for trend)**	**708**	**p = 0.04**

Table 1-2: Table demonstrating relationship between BMI and relative risk of death from PCa (7).

Although the BMI categories are different in this study (possibly due to a lower incidence of obesity in Sweden compared with USA) there again appears to be an increase in the incidence of prostate cancer as well as mortality from prostate cancer with increasing BMI. With regards to the incidence of prostate cancer this trend is not statistically significant, however with mortality, it is. For example the risk of dying from prostate cancer if your BMI is >26.2 is 40% higher than if your BMI is < 22.1. This might be considered by some to be a modest increase, but considering that prostate cancer is the second commonest cause of cancer death in the UK, this means that obesity is potentially a significant factor in all prostate cancer deaths. Another Scandinavian study, this time from Norway, is probably the largest prospective cohort study into BMI and prostate cancer (8). Similar to the Andersson

study patient recruitment was based on large-scale recruitment to a programme aimed at health promotion. During the time in question there was a national programme in Norway aimed at the detection and screening of Tuberculosis. Attendance was compulsory over the age of 15 and 1.7 million people were recruited (85% of the total population) to this study. Weight and height was recorded independently in a standardised way and 951 466 men were eligible for study. In total 951 459 men (seven lost to follow up) were followed for an average of 21 years until prostate cancer diagnosis, emigration, age 100, death or June 2001, whichever was earliest. Records were linked to the national cancer and death registers respectively. The mean age of the cohort was 44.5 years and as expected there was in increase in the observed incidence of histologically verified prostate cancer with increasing age at measurement (the earliest weight measurement for each man). This study only reports the incidence of prostate cancer and not prostate cancer mortality which I feel is a shortcoming of this study particularly as it has such a large cohort. In this cohort there is a statistically significant increase in prostate cancer incidence with increasing BMI $\geq$ 23.5 up to, but not including a BMI of 35 as demonstrated by the following table (Table 1-3).

BMI (Kg/m^2)	Number of cases of Prostate cancer	Relative Risk (Incidence) (95% CI)
22.50 – 23.49	3766	1.0
23.50 – 24.99	6983	1.04 (1.00 – 1.09)
25.00 – 27.49	10 001	1.07 (1.03 – 1.11)
27.50 – 29.99	4523	1.07 (1.02 – 1.12)
30.00 – 32.49	1453	1.09 (1.03 – 1.16)
32.50 – 34.99	370	1.15 (1.03 – 1.28)
≥ 35.00	100	0.93 (0.76 – 1.13)
	27 196	**p < 0.001**

Table 1-3: Table demonstrating relationship between BMI and relative risk of incidence of PCa (Engeland et al., 2003).

It is unclear why the relative risk of being diagnosed with prostate cancer is lower in the group with a BMI ≥ 35.00. It must be remembered however that the study only includes *pathologically* confirmed prostate cancer cases. Participants in this group (BMI ≥ 35.00) are technically classified as severely obese or very severely obese and may therefore have other significant co-morbidities such as DM or cardiovascular diseases such as ischaemic heart disease. In this group of patients it might not be deemed appropriate to perform a prostate biopsy when prostate cancer is suspected due to their limited life-expectancy. Performing prostate biopsies in men with limited a life-expectancy is not normal practice. These patients may well have a diagnosis of *clinical* prostate cancer and be receiving treatment or being monitored but not be labelled as prostate cancer according to this study. When analysed by attained age (age at the end of the study) it was between the ages of 50-59 where the greatest increase in prostate cancer incidence was demonstrated. Beyond the attained age of 59 there appeared to be no significant increase in prostate cancer incidence with increasing BMI. The table below demonstrates the incidence of prostate cancer in the attained age group 50-59:

BMI (Kg/m^2)	Relative Risk (Incidence)	(95% CI)
< 18.50	0.82	0.41 – 1.65
18.50 – 24.99	1.00	Referent
25.00 – 29.99	1.13	1.02 – 1.25
≥ 30.00	1.58	1.29 – 1.94

Table 1-4: Table demonstrating relationship of increasing BMI with PCa incidence in participants aged 50-59.

Mean BMI tends to reduce after the age of 59(9) which in some way may explain the lack of an increased risk of prostate cancer diagnosis with increasing BMI after the age of 59 in this study.

Obesity or more specifically increased visceral fat levels appears to be a risk factor for PCa as quantified by computed tomography (CT) scan (10). In 63 individuals with histologically-confirmed PCa total visceral fat (VF) area at the level of the fourth lumbar vertebrae was significantly higher than 63 age/BMI-matched healthy individuals (VF area (cm^2) 324.7 ± 145.6 vs 177.4 ± 88.4). Interestingly there was no difference in subcutaneous (SC) fat areas between the two groups SC area (cm^2) 184.5 ± 85.6 (PCa) vs 156.9 ± 57.6 (benign) as well as BMI. There was no difference demonstrated in disease stage between differing fat indices in this study. The increased levels of visceral fat in these patients suggests with prostate cancer suggests that it is not just how much extra fat a particular patient has but where that fat is located. The presence of more adipose tissue in the abdominal (and possibly pelvic) cavity may well lead to an increase in the local levels of metabolically active chemicals, termed adipokines which are thought to act in an autocrine and paracrine fashion in and around the prostate gland and may lead to an increase in prostate cancer risk. It is believed that the use of CT scanning is a more reliable and reproducible way of assessing a person's adipose tissue levels rather than relying on weight, BMI or waist to hip ratio (WHR) measurements which otherwise may lead to error. A review of the data published in 2007 suggests that obesity is associated with a reduced risk of being diagnosed with "nonaggressive" PCa but an increased risk of being diagnosed with

"aggressive" PCa (11). The authors of this article suggest three possible reasons for the inherent difficulties in the diagnosis of PCa in obese individuals:

- Difficulty in performing a thorough digital rectal examination
- Apparent lower PSA levels, due to either:
    - Lower testosterone levels
    - Increased plasma volume- Obese men tend to have lower PSA levels due to the effect of higher plasma circulating volumes and subsequent haemodilution (12).
- Larger prostate volumes- Increased BMI is associated with a larger prostate volume in men with prostate cancer in a study analysing 16 325 radical prostatectomy specimens (13).

These three factors mentioned above mean that PCa is potentially diagnosed at a later and more advanced stage in obese individuals than in non-obese individuals. The apparent lower levels of PSA found in obese individuals leads to the potential under-detection of PCa in obese individuals as a significant proportion of PCa diagnosis is down to PSA testing. The likelihood of accurately sampling prostate cancer is reduced in a larger prostate compared to a smaller prostate even if the same volume of disease is present. In this situation prostate biopsy is analogous to hunting for a "needle in a haystack." Two men may have exactly the same volume of tumour in their prostate but if one has a larger prostate, due to sampling inaccuracies the man with the larger prostate is less likely to be diagnosed. This is a sampling error and a known problem with current prostate biopsy strategies. It is unlikely however that the increased mortality seen in obese individuals with PCa is only down to the three factors mentioned above. Recent evidence suggests however that BMI is also associated with *increased* tumour volume in patients undergoing radical prostatectomy. In a study of 1275 radical prostatectomy specimens mean tumour volume in severely obese patients was 9.2ml compared with 5.0ml in normal weight patients (14).Obese patients may therefore present with relatively advanced disease at a later stage compared with leaner

individuals. With larger tumour volume, patients undergoing a radical retro-pubic prostatectomy (RRP) are more likely to have tumours that have breached the prostate capsule (T3) or have positive surgical margins rather than be organ confined (T2). Both of these factors are associated with a poorer outcome following surgery. Adipose tissue is now considered an organ within its own right with various autocrine, paracrine and endocrine functions and it is thought to play a key role in prostate carcinogenesis in obese individuals.

After surgery obese individuals are also at an increased risk of progressing to metastasis despite androgen deprivation therapy (ADT) and have a slightly higher incidence of castrate-resistant disease and death from prostate cancer (15). This study included men who underwent a RRP over a period of 21 years at five hospitals in USA as part of the SEARCH database. Excluding those with missing data 287 men were identified who were treated with early continuous ADT following RRP. Hormone-refractory prostate cancer (HRPC) was defined as a rise of 25% from the ADT PSA nadir *and* a PSA increase of ≥ 2ng/ml. Development of metastasis was confirmed after reviewing the medical notes and available imaging. Of the 287 men 44 (15.3%), 34 (11.8%) and 24 (8.4%) developed HRPC, metastatic disease and died from prostate cancer respectively. There were no differences between the groups (categorised by normal, overweight and obese or above by BMI) in terms of age, race, PSA characteristics or pathological features, other than Gleason score. Gleason score is the system by which PCa is graded pathologically and is a measure of the aggressiveness of the disease (16). Overweight and obese men had significantly higher Gleason scores at baseline i.e. less Gleason 6 or below and more Gleason 7 or above. Overweight ($25 \geq BMI < 30$) and obese ($BMI \geq 30$) men were three and five times more likely, respectively to develop metastasis than normal weight ($BMI < 25$) men. On multivariate analyses there was a slight increased risk of prostate-cancer specific mortality with increasing BMI but this was not significant, possibly due to the small number of deaths in the study (n = 24). Several theories are postulated for these findings in this study. Firstly, obese men receiving ADT may not be getting therapeutic levels of the drug, due to increased

plasma volume, to adequately suppress testosterone, the main principle of ADT. Secondly it may be the case that tumours in obese men are already acclimatized to low testosterone levels and are therefore more inherently aggressive as they are already used to developing in a low testosterone environment pre-diagnosis. Thirdly it may be that adipokines such as Insulin-like growth factor (IGF)-1 and leptin are responsible for poorer outcomes and the development of metastatic disease in PCa. Limits of this study include its retrospective nature and the fact that data regarding height and weight was not collected in a uniform and standardised way. There is also evidence that weight gain of $\geq$ 2.5Kg in the year prior to a RRP for PCa is associated with an increased risk of biochemical recurrence on multivariate secondary analysis, whereas weight loss $\geq$ 2.5Kg is not (17).

1.4 Adipokines and prostate cancer

Adipokines or adipocytokines are a group of chemicals that are actively secreted by white adipose tissue (WAT) and have a diverse array of effects on multiple cell processes in the body.

Adipokines identified so far include:

- Leptin
- Interleukin 6 (IL-6)
- Vascular Endothelial Growth Factor (VEGF)
- Tumour Necrosis Factor α (TNF-α)
- Adiponectin
- Visfatin (pre-B cell colony-enhancing factor 1)
- Chemerin

Adipokines exert their effects through various autocrine, paracrine and even endocrine pathways. It is thought that adipokines may play a pivotal role in the development of obesity-related cancer. The role that adipokines play in the pathogenesis of PCa is the subject of a review article published in 2007 (18). It is thought that adipokines may in part explain the increased mortality seen in obese individuals with PCa. It may be that adipokines exert their effects in two main ways. Firstly, the active secretion of adipokines, from, for example the retro-pubic fat pad may be acting locally in an autocrine or paracrine fashion. The pro-angiogenic or pro-proliferative properties of adipokines may *directly* initiate or perpetuate prostate carcinogenesis on the prostate gland itself. Secondly adipokines may act in an endocrine way, being secreted in to the bloodstream and initiating their effects via specific cell-surface or nuclear receptors on or within the prostate cells themselves. Our research collaborative has previously published work on the effects of the adipokines Leptin, Adiponectin and Visfatin in PCa.

Serum Leptin levels are positively correlated with BMI, with concentrations of approximately 100nM found in obese individuals. 0.01, 1 and 100nM Leptin causes a significant increase in androgen-independent prostate cancer (PC3) cell proliferation after 48 hours with respect to basal (19). 1nM Leptin also causes a significant increase in PC3 cell proliferation after 24 hours treatment with respect to basal. No effect on cell proliferation was found when androgen-dependent prostate cancer (LNCaP) cells were treated for 24 and 48 hours with 0.01 - 100nM leptin. In 199 men undergoing a RRP for PCa, those with higher volume disease (> 0.5cc, n = 151) had significantly higher serum leptin levels (7.04ng/ml vs 4.65) than in those men with low volume disease (≤ 0.5 cc, n = 48) (20). Another study with a relatively small number of prostate cancer subjects (n = 21) found significantly higher leptin levels in cancer patients (27.33ng/ml) compared with men with BPE (16.96) or controls (17.55). Higher levels of PSA are found in the patients with prostate cancer and so these authors conclude that leptin and PSA levels are correlated (21). Leptin promotes activation of protein kinase B (PKB/AKT) in DU145 prostate cancer cells as well as extracellular-signal-regulated kinase (ERK) in PC3 cells in a dose and time – dependent manner (22). AKT and ERK are key proteins in cell metabolism and are often up regulated in many cancers which will be discussed in more detail later in chapter 5.

Adiponectin circulates abundantly in human plasma and unlike most other adipokines levels are *inversely* correlated with BMI with lower levels being found in obesity-related conditions such as insulin resistance and type 2 diabetes (T2D). One of the earliest studies reporting a possible link between Adiponectin levels and PCa was published in 2005 (23). When comparing serum adiponectin levels between 30 men with PCa, 41 men with clinically benign prostatic obstruction and 36 healthy volunteers, men with PCa had significantly lower Adiponectin levels (5.3µg/ml) compared to those with benign enlargement (14.5µg/ml) and healthy controls (16.2µg/ml). There were no differences between the three groups with regards to their age, BMI, lipid parameters or fasting blood glucose levels. This suggests that the differences in adiponectin levels between the three

groups could not be attributed to these parameters alone. In a further sub-analysis patients with organ-confined disease and those with advanced PCa (those with PCa extending into the prostatic capsule or further (≥T3)) were compared. Those with advanced disease had significantly lower levels than those with localised disease (4.7μg/ml vs 6.0μg/ml). In the PCa group men with a higher Gleason grade, representing poorer cellular differentiation also had significantly lower adiponectin levels. What is perhaps more clinically important in PCa is not the risk of developing the disease but the risk of death from PCa. The ability to accurately predict the risk of a particular patient dying from PCa is an important characteristic of a PCa marker and this is where adipokines could potentially play an important role. This was the aim of a case-control study nested in the Physician's Health Study conducted between 1982 – 2000(24). 654 cases of PCa were detected during the study and these were matched against 644 randomly selected healthy controls. Blood samples were drawn prior to inclusion into the study and adiponectin levels were calculated by competitive radioimmunoassay. There was no association between adiponectin levels and risk of the incidence of PCa but lower adiponectin levels were associated with a greater risk of developing high risk PCa as well as lethal PCa. Burton et al., published a study looking at the role of Adiponectin and leptin levels nested within the ProtecT study. The ProtecT study is population-based study within the UK hoping to assess which is the best treatment for localised prostate cancer (surgery, radiotherapy or active surveillance) in a PSA-screened population (25).So far over 100 000 men (aged 50-69) have taken part from 9 centres across the United Kingdom. Medical and demographic data was collected for each patient including height weight and a blood sample was collected several weeks prior to randomisation into a particular arm of the study. Overweight or obese men tended to have lower Adiponectin levels and this was associated with having locally advanced or metastatic PCa. In all men or in normal weight men this association with adiponectin levels was not demonstrated. No association was demonstrated between leptin levels, BMI or risk of advanced prostate cancer. The Adiponectin receptors: Adipo-R1 and Adipo-R2 are expressed in PC3 and LNCaP cells as well as in benign and malignant human prostate tissue

for the first time suggesting a possible role for adiponectin in prostate carcinogenesis (26).p53 messenger ribonucleic acid (mRNA) expression is also significantly increased in PC3 cells treated with 100nM Leptin combined with 0.01 and 100nM fAd (full-length adiponectin) after 24 and 4 hours respectively (19).

Our research group has published the only available data on the potential role of the adipokine visfatin in PCa. In-vitro studies have demonstrated that visfatin (pre-B cell colony-enhancing factor 1) causes a highly significant increase in PC3 cell proliferation after 24 hours at the levels 200-400ng/ml (27). Visfatin also caused increased expression of matrix metalloproteinases (MMP) -2/9 (crucial proteins in metastasis) mRNA and protein expression in PC3 cells further strengthening a potential role for adipokines in obesity-driven prostate cancer.

1.5 Chemerin

Chemerin is a 137 amino acid (aa),16 kDa protein and is secreted as pro-chemerin as an inactive pre-cursor (143 aa, 18 kDa) (28). Chemerin is activated when a 6-aa peptide is cleaved at its C-terminus by serine proteases. It has also previously been known as Tazarotene-Induced Gene (TIG)-2 or Retinoic Acid Receptor Responder protein 2 (RARRES2). Retinoids are biological compounds which exert their effects by acting on nuclear receptors which belong to a superfamily of steroid/thyroid steroid hormone nuclear receptors. The gene TIG2/RARRES2 is located on chromosome/location7q36.1 (HUGO Gene nomenclature committee). The name TIG2 was coined after it was demonstrated that in lesional psoriatic skin lesions its expression was up regulated by the anti-psoriatic synthetic retinoid tazarotene (29), as confirmed by northern blot analysis. In non-lesional psoriatic skin lesions TIG2 is also expressed at high levels. Wittamer et al., hypothesise that the chemerin receptor: ChemR23 is similar in structure to other receptors that are specific for chemokines and attract Antigen Presenting Cells (APC) (30). It is thought that an immune response is mediated through the ChemR23 receptor recruiting APC via an, at the time unidentified chemoattractant protein. In order to prove this hypothesis chemerin was isolated from various human inflammatory liquids. The activity of chemerin was assessed through the ability of the fractions to activate ChemR23. Chemerin was isolated from the synovial fluid of patients with arthritis and osteoarthritis and from the ascitic fluid of patients with ovarian carcinoma and ovarian hyperstimulation syndrome (OHS) using this method. Chemerin/ChemR23 has since been found to be expressed in human chondrocytes which may explain how chemerin was found to be present in human synovial fluid (31). It is possible that chemerin signalling acting as a ligand to the ChemR23 receptor may play a key role in the inflammatory process taking place in human synovial fluid via chondrocytes. The chemerin receptor (ChemR23) has been isolated from monocyte-derived dendritic cells and macrophages – this being one of the earliest reports of this specific G protein-coupled receptor (GPCR) in the literature (32). GPCR's are receptors that are involved in multiple

cellular processes and mediate their actions by activation of one or more guanine nucleotide-binding regulatory proteins (G proteins) (33). GPCR's share considerable structural homology, despite their varied biological actions, characteristically consisting of seven hydrophobic stretches of 20-25 amino acids, connected by alternating extracellular and intracellular loops. Activation of the GPCR is an energy-dependent process leading to dissociation of the α and βγ subunits of the G protein resulting in activation of their respective downstream signalling cascades (34).

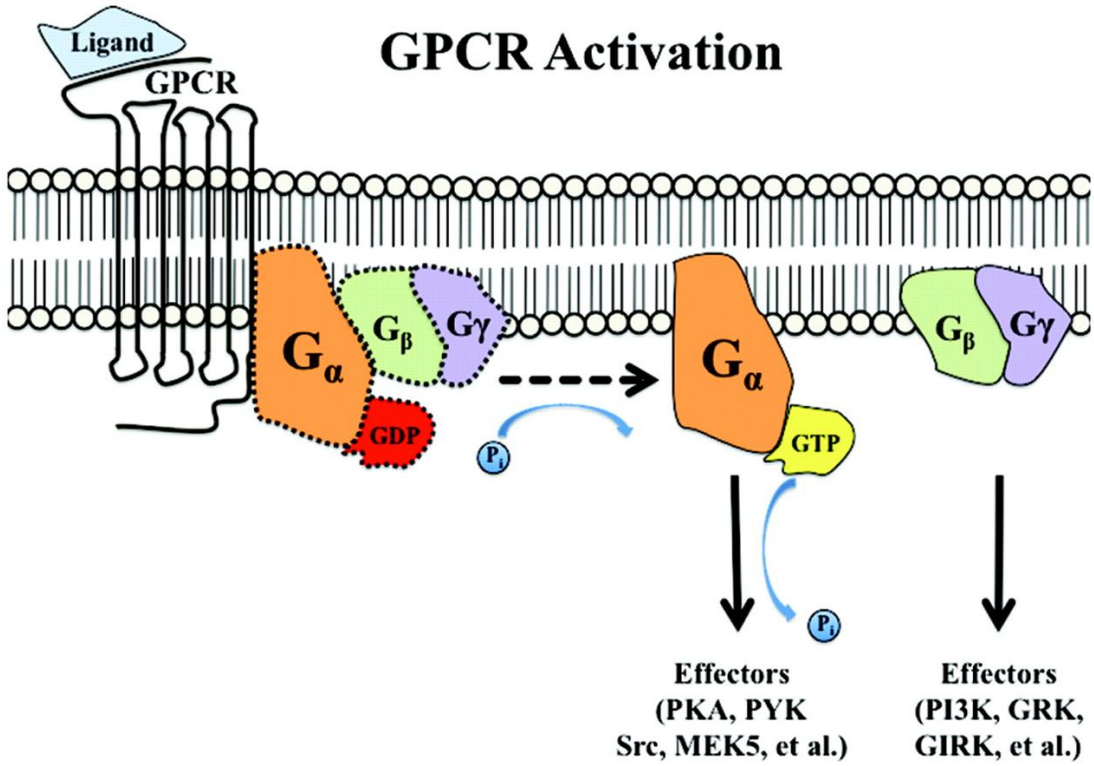

Figure 1-3: Diagram demonstrating schematic GPCR structure with subsequent mechanism of activation (34).

It is also reported that ChemR23 transcripts are detected in CD4+ T lymphocytes and its location as detected by radiation hybrid mapping is chromosome 12, lociq21.2–21.3. The ChemR23 receptor appears to play a key role in the recruitment of inflammatory cells (such as macrophages and T lymphocytes) and works as a co-receptor for several Simian Immunodeficiency Virus (SIV) strains as well Human Immunodeficiency Virus (HIV) 1.

The term “chemerin receptor” (ChemR) is synonymous with the terms ChemR23 and Chemokine-Like Receptor 1 (CMKLR1). Both have been proven to have a key role in acting as co-receptors for HIV 1/2 as well as SIV (35) in their recruitment of inflammatory cells. This confirms that even though the receptors have different names they are structurally, and more importantly functionally identical and can be treated the same way. Circulating pro-chemerin has also been reported to be activated by serine proteases factor XIIa, VIIa and plasmin, key products of the fibrinolytic and coagulation cascades. The process by which chemerin activation occurs is by proteolysis of the carboxyl-terminal peptide part of the pro-chemerin molecule. Three endogenously active isoforms of human chemerin are isolated during this study – all requiring cleavage of the carboxyl-terminal peptide part of pro-chemerin for activation. Tissue injury and bleeding therefore stimulates chemotaxis of inflammatory cells such as macrophages expressing the chemerin receptor: CMKLR1 (36) to the inflammatory process. Chemerin activation can also be stimulated by an allergic response via the release of tryptase from mast cells. An infective response caused by the release of elastase from neutrophils also leads to the activation of chemerin from pro-chemerin. Chemerin upon activation therefore, acts as an important mediator of immunity by recruitment of immune cells to the innate and adaptive immune system. The first report of chemerin as an adipokine in the literature was made by Goralski et al., as recently as 2007 (37). Whereas in previous studies mentioned earlier it has been established that chemerin is present in inflammatory exudates such as ascitic fluid, synovial fluid and in peripheral blood it is not clear what is the site or sites of chemerin production. The hypothesis proposed by Goralski et al., is that chemerin plays a key role in adipocyte function and adipogenesis in WAT. The following samples were used for detection of chemerin and its receptor CMKLR1: male adult mice, human samples and 3T3-L1 preadipocytes. It is demonstrated that in male adult mice chemerin mRNA expression is highest in WAT, liver, and placenta and the expression of the chemerin receptor: CMKLR1 mRNA was highest in WAT with lower levels of expression found in lung, heart and placenta. Regarding expression of

chemerin in adipose tissue the following sites were used to determine differing levels of chemerin expression (top = highest expression):

- Mesenteric
- Perirenal
- Epididymal (visceral)
- Inguinal
- Brown Adipose Tissue (BAT) ($p \leq 0.05$)

Although levels of chemerin mRNA expression between different sites of WAT differed these were not statistically significant. Levels of chemerin (and CMKLR1) mRNA expression in BAT were significantly lower ($p \leq 0.05$) than WAT. In the human samples it is demonstrated that the highest levels of chemerin mRNA expression were found in subcutaneous WAT. The highest levels of CMKLR1 mRNA expression were found in placenta, subcutaneous WAT and liver. Using this above expression data it is said with reasonable certainty that WAT is the main source and a target for chemerin signalling. To further strengthen this theory it was noted that upon differentiation of 3T3-L1 preadipocytes in adipocyte media, expression of chemerin and CMKLR1 mRNA was significantly increased after 8 days and 13 days respectively ($p \leq 0.05$). These findings are in keeping with data published elsewhere regarding chemerin/ChemR expression in 3T3-L1 cell differentiation. A significant rise in chemerin mRNA is detected as early as day 3 post differentiation in adipocytes treated with troglitazone (5 μM) and ChemR mRNA as early as day 5 (38). Differing concentrations of mouse and human chemerin (0.2 – 10nM) were also noted to temporarily and reversibly stimulate p42 (ERK1) and p44 (ERK2) mitogen activated protein kinases (MAPK) phosphorylation in mature murine and human differentiated adipocytes (37) with maximum activation being noted at 5 minutes. The strength of ERK1/2 phosphorylation appeared lessened at the higher concentrations of chemerin used, this may suggest an element of desensitisation of this signalling pathway at higher concentrations of chemerin. Treatment of differentiated human 3T3-L1 cells with

murine recombinant chemerin (10^{-8}) also caused reversible phosphorylation of ERK1/2 with maximum activation noted after 3 minutes before falling to basal levels again after 5 minutes (38). It has been demonstrated that MAPK signalling may play a key role in adipogenesis and lipolysis during preadipocyte differentiation (39). This interaction of chemerin, MAPK and adipogenesis in adipose tissue and adipocytes confirms chemerin as an adipokine in its own right. There appears to be a clear emerging role for chemerin in physiological adipose tissue function and possibly pathological conditions involving adipose tissue, such as obesity. In another study looking at chemerin/CMKLR1 expression in Psammomys obesus (*P. obesus* - an animal model for obesity and T2D) expression of chemerin was demonstrated to be highest in WAT, liver and kidney with CMKLR1 expression highest in WAT and lung (40). These findings are similar in C57BL/6J mice where chemerin mRNA expression was highest in adipose tissue, liver and lung and ChemR mRNA expression was highest in adipose tissue, lung, kidney and heart (38). In mesenteric fat chemerin mRNA expression appeared to be higher in animals with impaired glucose tolerance (IGT) and T2D in the *fasted* state compared with CMKLR1 mRNA expression where expression in these two conditions appeared to be higher in the *fed* state (40). In C57BL/6J mice fed a high fat diet, levels of chemerin/ChemR mRNA expression are significantly higher in all sites of adipose tissue (subcutaneous, perirenal, mesenteric and epididymal) compared to mice fed a normal diet (38).Upon isolating adipocytes and stromal vascular cells from the mesenteric adipose tissue of *P. obesus* it was also demonstrated that chemerin mRNA expression is significantly higher in adipocytes compared with stromal vascular cells in animals with normal or impaired glucose tolerance.CMKLR1 gene expression however, appears to be similar in both adipocytes as well stromal vascular cells in either normal or impaired glucose tolerance states in these animals (40).C57BL/6J mice fed either a normal or high fat diet demonstrated that chemerin/ChemR mRNA expression is significantly higher in adipocytes than stromal vascular cells isolated from mesenteric adipose tissue (38). It is thought that increased visceral fat accumulation is more likely to be associated with features of the metabolic syndrome (MetS) such as insulin resistance, rather than subcutaneous fat accumulation (41).

With this in mind it is interesting to note that in normal glucose tolerance (NGT) animals, levels of chemerin mRNA expression are significantly higher in visceral adipose tissue when compared to SC adipose tissue. In T2D animals levels of chemerin expression in visceral fat were also significantly higher than in NGT animals. Regarding CMKLR1, expression in visceral adipose tissue was significantly higher in animals with IGT and T2D compared to animals with NGT. As proven previously this study reports that as adipocytes differentiate and mature the levels of chemerin gene expression increase significantly confirming that it is most likely the adipocytes in adipose tissue that produce chemerin. Features of the MetS (a clinical syndrome that leads to an increased risk of developing DM and cardiovascular disease), such as fasting insulin and glucose levels, height, weight, lipid profile and blood pressure in 256 individuals aged between 35–65 years from Mauritius were assessed during this study. Serum chemerin levels were assessed using an enzyme-linked immunosorbent assay (ELISA), 142 individuals had NGT and the rest (114) had T2D. No difference was demonstrated in serum chemerin levels between the two groups (NGT, chemerin = 249ng/ml ± 71, T2D, chemerin = 250ng/ml ± 67). In NGT participants chemerin levels were significantly higher in those individuals with a BMI > 30 (296.5 ± 61.2 ng/ml) compared to those with a BMI < 25 (222.7 ± 67.1 ng/ml). Given the fact that chemerin is generally produced by adipocytes you may well expect that the higher a person's BMI (and generally the higher their fat mass) the higher their chemerin levels would be expected to be. Strong correlations between age, sex and chemerin levels were also detected when linear regression analysis was performed. After correcting for age and sex, chemerin levels were significantly associated with the following body fat and MetS parameters in NGT individuals:

- BMI
- Fat mass
- Weight
- WHR
- Fasting glucose levels

- Fasting insulin levels
- Plasma triglycerides levels
- Blood pressure

The data presented here confirms chemerin as a novel adipokine with a potentially pivotal role in obesity and development of MetS. Chemerin can be used as an independent marker for the development of the Mets (42). This cross-sectional study recruited a similar number of patients to Bozaoglu et al., (n = 236), with 55 non-obese healthy individuals, 122 deemed at risk of developing and 59 subjects with confirmed MetS. Patients deemed at risk of developing MetS were those being treated for, or being monitored for some element of the MetS e.g. dyslipidaemia (raised triacylglycerides or low high-density lipoprotein (HDL)), IGT, hypertension or obesity. Those with established DM were excluded from the study. Those with established MetS had significantly higher chemerin levels compared to those at risk of developing MetS and healthy subjects (**285.0** ± 80.70μg/l vs **228.9** ± 41.10 μg/l vs **192.5** ± 25.7 μg/l). In line with previous studies there a positive correlation between age and chemerin levels. There was found to be a *positive* correlation between serum chemerin levels and the following parameters in this study:

- Glucose
- Triacylglycerides
- Systolic and Diastolic blood pressure
- Number of risk components of the MetS

There was found to be a *negative* correlation between serum chemerin and HDL-cholesterol levels and no association (either positive or negative) was found between BMI, waist circumference (WC) or Insulin sensitivity check index (Quicki) and chemerin levels. When patients were stratified according to the number of individual components of the MetS they fulfilled and their serum chemerin levels there was a significant positive correlation confirmed by a chi squared test. This study strengthens the position that chemerin as an

adipokine is associated with several features of the MetS and therefore may be a key peptide in its pathophysiology. The process by which these processes happen is beyond the remit of this particular paper. Chemerin has also been found that in patients with the MetS, those with CAD are more likely to have higher chemerin levels (43). In 112 patients undergoing coronary angiography those with MetS (diagnosed according to Adult Treatment Panel III) who had confirmed CAD (stenosis >50% in at least one major coronary artery) had significantly higher chemerin levels (133.08μg/l) than those without CAD (111.56μg/l). Individuals with MetS but without evidence of CAD also had higher chemerin levels than 52 healthy controls (95.07μg/l). Understandably the difference between the chemerin levels in the healthy controls and the subjects with MetS and CAD was profound. Multiple stepwise regression analysis showed, in keeping with previously published data that serum chemerin levels were significantly associated with BMI and C-reactive protein (CRP). This data strengthens the position of chemerin being associated with a clinical condition (MetS) but also of a hugely important clinical entity CAD. The ability of chemerin to predict the presence of a condition such as CAD in a population at risk (i.e. those with MetS) is potentially of huge importance in managing these patients effectively. The positive correlation with CRP once again strengthens the hypothesis that obesity is a pro-inflammatory state and that the complications of obesity are possibly due to the up-regulation of pro-inflammatory cytokines such as chemerin.

Most of the adipose tissue expression studies up until this point have reported the amount of chemerin expression in mouse models. Tan et al., published data using human samples with polycystic ovarian syndrome (PCOS) and the amount of chemerin expression in human adipose tissue (44). Women with PCOS were recruited to this study and were matched against the same number of healthy control subjects. PCOS is an endocrinopathy characterised by menstrual dysfunction, IGT and obesity(45). Circulating chemerin levels were significantly higher in PCOS subjects than in healthy controls (6.02ng/ml vs 2.62ng/ml. Interestingly there was no significant difference in the levels of other circulating adipokines:

leptin or adiponectin Chemerin mRNA and protein expression was significantly higher in PCOS subjects in subcutaneous and omental adipose tissue deposits than in the respective adipose tissue deposits of healthy controls. Women with PCOS treated with the oral hypoglycaemic drug: metformin for a minimum of six months were found to have significantly lower chemerin levels (6.36 ng/ml vs 2.19 ng/ml. These results suggest that chemerin levels are closely linked to the degree of metabolic control that patients with an endocrine disorder have over their condition. Chemerin could therefore be used as a biochemical marker in women with PCOS and may reflect their metabolic control of their condition. Chemerin appears to stimulate capillary development in an *in vitro* angiogenesis model (46). Formation of capillary-like structures was increased 1.34-fold at 0.3 ng/ml chemerin, and by 1.38-fold by both 1 and 3 ng/ml chemerin. Interestingly no increase in capillary-like structure formation was detected at 10ng/ml chemerin – possibly to due toxic effects of chemerin at these levels. The angiogenic effects of chemerin appear to be mediated through the p42/44 MAPK pathway. The dual specificity mitogen-activated protein kinase kinase (MAP2K1/MEK1) inhibitor: PD 98059 in combination with chemerin significantly reduced the number of microtubule junctions, number of tubules, total tubule length and total tubule area in an angiogenesis model. As adipose tissue is a highly vascular organ its development is highly dependent on processes such as angiogenesis. The findings that chemerin plays a key role in angiogenesis has been corroborated by data from our own research group. ChemR23 mRNA and protein was demonstrated to be present in human microvascular endothelial cells (HMEC) and human umbilical vein endothelial cells (HUVEC) (47). The presence of the chemerin receptor: ChemR23 in endothelial cells is an important finding to prove a potential role for chemerin in human angiogenesis. Human chemerin causes a significant dose-dependent increase in proliferation, capillary tube formation and migration in HMECs. The greatest effect on proliferation was noted at 0.1nM chemerin and for capillary tube formation and migration at 30nM chemerin. 0.5nM VEGF was used as a positive control in these experiments. Chemerin also stimulates phosphorylation of p38 MAPK, ERK1/2 MAPK and AKT – signalling pathways in a time

(2-30 minutes) and a dose (0.01-30nM) dependent manner. This data presented here confirms that as an adipokine, chemerin plays a key role in a number of physiological processes – angiogenesis being a key pathway under direct control by chemerin. Evidence has arisen more recently which suggests that obesity is associated with chronic inflammation and subsequent increased circulating levels of cytokines such as TNFα or IL-6 (48). It may be that these increased levels of cytokines are a response to obesity and play a part in the development of obesity-related disease such as insulin resistance and T2D. It may be that increased cytokine levels lead to the development of obesity-related conditions and the production of WAT. The ways these inflammatory mediators interact with each other is undoubtedly complex and are probably controlled by a mixture of positive and negative feedback mechanisms. To illustrate this it has been demonstrated that in 3T3-L1 adipocytes TNFα causes a dose (0.1 – 20ng/ml) and time (2-24 hour) dependent increase in chemerin mRNA (49). TNFα was also found to increase the apparent amount of chemerin detected in serum-free adipocyte cell culture after 24 hours treatment in a dose-dependent manner. This apparent stimulation of increased chemerin production *in vitro* has been replicated *in vivo* using wild-type (B6/129SF2/J) injected with intra-peritoneal TNFα with significantly increased serum chemerin levels detected as soon as 12 hours after treatment. The increased amount of chemerin in these animals at this time point (12 hours) was confirmed by the ability of the serum to activate CMKLR1. No increase in chemerin *expression* was found in bone marrow stromal cell (BMSC)-derived primary adipocytes after TNFα treatment suggesting that the increased serum levels of chemerin detected was due to increased *secretion* of the adipokine rather than its expression. Measuring serum chemerin levels at multiple time points throughout a 24 hour period enables the authors of this study to report that chemerin levels oscillate throughout the day and roughly correspond to a night and day pattern. This raises the possibility that chemerin plays an important role in either sleep/wake cycles or eating patterns in mammals.

In studies thus far most of the data pertaining to the expression of chemerin and ChemR (either mRNA or protein) has involved the use of animal models to ascertain in what organs and tissues its expression is highest. In order to assess the expression of chemerin from the liver a study was conducted by Weigert et al., where venous samples were taken from the portal, hepatic and systemic veins in forty-four patients with liver cirrhosis undergoing a transjugular intrahepatic portosystemic shunt (TIPS) procedure for complications of their liver disease (50). Six healthy subjects undergoing liver surgery for metastatic cancer but with normal liver function. In the cohort of patients with cirrhosis there was a significant rise in chemerin levels detected in the hepatic vein compared with the portal or systemic venous chemerin levels (values not given). Interestingly in the six controls no elevation was demonstrated in the chemerin levels in the hepatic vein compared with the portal, systemic venous, or indeed the arterial blood samples. If the liver was the main site of chemerin production rather than adipose tissue then one might expect the hepatic vein chemerin levels to be higher in these subjects too. On the other hand if adipose tissue is the main site of chemerin production in humans then one might have expected that, in the healthy subjects the chemerin levels would be significantly higher in the systemic venous blood compared to the arterial blood which wasn't demonstrated either. It may be that the difference is so subtle that it is undetectable. The capillary bed in adipose tissue is much larger and much more spread out than that of the liver and so it may be difficult to demonstrate a significant rise in chemerin levels between the arterial and venous circulation. Clearly further work is needed in humans in this area to ascertain the most prevalent sites of the production of chemerin. In another cohort used by this study of normal (BMI $\leq$ 25) and overweight (BMI > 25) male participants and those with T2D showed that chemerin levels were significantly higher in T2D and overweight) compared with normal weight participants. There was also a positive correlation between chemerin levels and BMI through all levels of BMI. Interestingly in the T2D cohort chemerin levels were significantly higher in those with a CRP > 5mg/l suggesting that possibly chemerin levels could also be associated with systemic inflammation, as previously mentioned and not just obesity. One of the only studies

comparing the relative expression of chemerin mRNA in human subcutaneous adipose tissue compared with visceral adipose tissue showed that expression of chemerin was significantly higher in subcutaneous than in visceral adipose tissue (51). Once again a positive correlation is demonstrated between log serum chemerin levels and BMI in this cohort of 97 men and women undergoing elective abdominal surgery. Interestingly the levels of chemerin mRNA expression were higher in subcutaneous and visceral adipose tissue in females when compared to males. This may be of particular interest to those studying the aetiology of obesity-related disease and even cancers in females. A significant negative correlation between log serum chemerin levels and chemerin expression in subcutaneous adipose tissue was not replicated in visceral adipose tissue. This suggests a possible negative feedback mechanism where high levels of chemerin expression in subcutaneous tissue lead to a reduced serum chemerin level. What the authors of this study fail to comment on is the BMI values or presence of T2D in this small cohort of subjects (n = 23). As discussed these two factors are likely to dictate the levels of chemerin expression in these samples one way or another.

As yet there is little data in the literature evaluating the role of chemerin, obesity and the development of cancer. As an adipokine there could potentially be a role for chemerin to be used as a tool for risk stratification in patients with cancer or indeed as an aid in diagnosis. Significantly raised chemerin levels have been demonstrated in the peripheral blood of patients with lung cancer compared with healthy subjects (52). In 42 patients with lung cancer their serum chemerin levels were significantly higher than in 31 healthy volunteers. There was no association between chemerin levels and any other clinico-pathological features in these patients. Although using only a few subjects this data raises the possibility of chemerin levels being used as a possible bio-marker for cancer diagnosis in the future. Venous blood tests are probably the most accepted invasive test that patients undergo when receiving medical investigations or treatment. Chemerin levels have been found at very high

levels (> 50ng/ml) in the ascitic fluid of patients known to be suffering with ovarian cancer and from a patient with liver cancer (30).

In this introductory chapter I have highlighted the evidence that exists currently between obesity, adipokines and prostate cancer. Adipokines, such as leptin and adiponectin have been shown to exert differing effects on prostate carcinogenesis in the past. My hypothesis is that chemerin also has a potential role in the development of prostate cancer. In order to evaluate the role that chemerin plays, basic cellular processes were assessed as well as the changes in key intracellular molecules in prostate cancer cells. I was also keen to explore the levels of chemerin expression, along with its receptor, in human prostate tissue as well as serum chemerin levels in adult men with prostate disease.

2 Materials and Methods

2.1 Cell culture and treatments

HAMS-F12 and RPMI-1640 culture media was purchased from Sigma-Aldrich (Gillingham, UK) and from Invitrogen (Paisley, UK) respectively. Heat inactivated 10% Fetal Calf Serum (FCS) was obtained from Biowest (Nuaillé, France). Recombinant human chemerin was purchased from R&D Systems (Abingdon, UK). Becton Dickinson (BD) Falcon™ 25/75/175cm^2 cell culture flasks were used for all cell cultures as appropriate.

PC3 and LNCaP human prostate cancer cell lines were obtained from the American Type Tissue Association (ATCC, Manassas, VA, USA) and cultured in HAMS F-12 and RPMI-1640 culture media respectively, supplemented with 10% FCS and 50units/ml penicillin G and 50µg/ml streptomycin sulphate. Cells were incubated in 175cm^2 cell culture flasks (BD Biosciences, Bedford, MA, USA) at 37^{0}C and 5% CO^2. In order to help prevent yeast contamination in the LNCaP cell line, RPMI-1640 media was supplemented with 5ml of 100x anti-mycotic (Gibco by Life Sciences) only instead of the penicillin/streptomycin supplement.

Once 80-90% confluent, cells were seeded onto six-well plates in preparation for treatment. Prior to treatments cells were pre-incubated with serum free media for at least 16 hours (overnight). Cells were then subsequently incubated with the appropriate treatment. Serum-free media was used for the cell treatments in order to minimise any potential effects of hormones and growth factors on chemerin receptor expression. Once the appropriate time point had elapsed the supernatants were removed from the well, placed in an appropriately sized vial (Eppendorf, Hamburg, Germany) and placed immediately on dry ice to achieve rapid freezing. 1ml of ice-cold Phosphate buffered Saline (PBS) was added to the well containing the adherent cells briefly and removed prior to lysing the cells with the appropriate solution (protein - 1x Radio-Immunoprecipitation Assay (RIPA) or RNA - Lysis

buffer (Sigma-Aldrich, St. Louis, MO, USA)). The wells were agitated with a pipette after the solution was added to ensure maximal cell lysis and therefore maximal protein or RNA extraction. The resultant lysate was immediately placed in an appropriately sized vial and placed immediately on dry ice prior to storage. All samples were stored at -20^{o}C until use.

No specific problems were encountered with PC3 cell culture during the time that I was using them. Due to their fast growth rate and the ease and speed at which they attached to flasks and plates they were relatively easy to use. This is probably due to the fact that they grow in a uniform monolayer when cultured correctly. The main problems that were encountered with the LNCaP cells were related to their slow rate of growth and the fact they tend to grow in clusters which tend to be quite fragile and can easily become detached if not handled correctly. Listed below are some of the strategies eventually used to help culture LNCaP cells successfully:

- During routine culture, culture medium was changed no more frequently than every 72 hours.
- Extra care was taken during medium exchange to be as gentle as possible so as not to detach the cells.
- Cells were washed with the RPMI medium (including FCS) itself prior to adding fresh RPMI medium rather than washing with PBS as this seemed to promote cell detachment.
- Every attempt was made to minimise vibration in the cell culture incubators.
- When seeding onto cell culture plates (6, 12 or 24 well) LNCaP cells were suspended in 2mls per well rather than 1ml (as for the PC3 cells).
- After seeding onto plates LNCaP cells were left for a minimum of 48 hours prior to any attempt being made at moving the cells or changing the medium prior to treatments.

2.2 RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted from cells using 200µl of a cell lysis buffer containing 10µl/ml β-Mercaptoethanol (BME) per well. Each well was agitated and scraped using a 1000µl pipette tip to ensure adequate lysis of the cells. The subsequent cell lysates and buffer was removed and placed in an Eppendorf tube on dry ice immediately for rapid freezing. For prostate **cells** RNA purification was performed using the GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) according to the protocol. For **human tissue** RNA was purified using a TRIZOL® RNA isolation protocol as described (53). RNA concentration was measured using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) using 1µl of the RNA solution. RNA was reversed transcribed into complementary Deoxyribonucleic acid (cDNA) using the *Precision*™ qScript Reverse Transcription kit (PrimerDesign Ltd, Southampton, UK) according to the manufacturer's guidelines. SYBR Green® quantitative real-time polymerase chain reaction (qRT-PCR) was performed on an ABI 7500 fast real time PCR using the primers listed below. All primers used (with the exception of AGR2) were obtained from PrimerDesign and were previously validated. AGR2 primers were used as previously described (54). PCR was performed using 4 µl (1:5 diluted) cDNA in 10 µl MasterMix (Applied Biosystems, Foster City, CA, USA), 4 µl water and 1 µl of mixed sense and antisense primers. For analysis, expression of genes of interest were normalised against the expression of the reference gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH is a commonly used reference gene used in qRT-PCR studies and appears to give satisfactory results in PCa (55). The relative mRNA levels were expressed as a ratio using the "2-Δct method" for comparing relative expression results between treatments in RT-PCR. All gene expressions were measured by RT-PCR, using 1 µg total RNA and oligo-dT primers as RT primers.

Primer	Sense	Anti-sense	Product size (base pairs)
Chemerin	5'-AGA CAA GCT GCC GGA AGA GG-3'	5'-TGG AGA AGG CGA ACT GTC CA-3'	252
ChemR23	5'-CAA CCT GGC AGT GGC AGA TT-3'	5'-AGC AGG AAG ACG CTG GTG AA-3'	153
P53	5′-GTG GAG TAT TTG GAT GAC AGA AAC-3′	5′-GTA GTT GTA GTG GAT GGT GGT AC-3′	102
Bcl-2	5′-GAG GTC ACG GGG GCT AAT T-3′	5′-GAG GCT GGG CAC ATT TCT G-3′	88
Bcl-2 L1	5'-CAC TTA CCT GAA TGA CCA CCT AG-3'	5'-GCA TTG TTC CCA TAG AGT TCC A-3'	84
BAX	5'-ATG GAG CTG CAG AGG ATG AT-3'	5'-CAG TTG AAG TTG CCG TCA GA-3'	101
AGR2	5'-CGA CTC ACA CAA GGC AGG T-3'	5'-GCA AGA ATG CTG ACA CTG GA-3'	73
GAPDH	5'-TGC ACC ACC AAC TGC TTA G-3'	5'-GAT GCA GGG ATG ATG TTC-3'	190

Table 2-1: Table demonstrating relevant sense/anti-sense primers used for qRT-PCR studies as per the MIQE Guidelines (56).

2.3 Agarose Gel Electrophoresis

A 1.5% Agarose gel was used for electrophoresis of the PCR products. Approximately 1L of 1x TAE buffer containing 4µl Ethidium bromide was used to run the PCR products. 4µl of 6X DNA loading dye (Fermentas) was added to each 20µl of PCR product per well and a 100bp DNA ladder (Invitrogen) was used to measure the distance of the PCR product to confirm the size of the band. After loading the PCR product into each well they were run from cathode to anode at 100v for approximately 40 minutes until the bands had separated

adequately. Bands were imaged using imaging software (GeneGenius Gel Imaging System, Syngene).

2.4 Western Blot Analysis (WBA)

Cells were lysed by adding 200µl of 1× RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) to each well. Protein lysates were quantified using the by a bicinchoninicacid (BCA) assay and were diluted using an appropriate amount of 2× sample buffer, Laemmli (Sigma Aldrich) to ensure standardisation of the protein concentration throughout the samples. Samples were heated at 95^{o}C for 15 minutes prior to use. Appropriate amounts of the protein were loaded onto 10% resolving gels and 4% stacking gels respectively (Geneflow, Fradley, UK). The proteins were transferred to 0.45µm Polyvinylidene fluoride (PVDF) transfer membranes (Immobilion®-P, Millipore, Billerica, MA, USA.) PVDF membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-Tween) and 5% bovine serum albumin (BSA) for 1 hour. Membranes were incubated overnight at 4^{o}C with the appropriate primary antibodies (Table 2-2) diluted in TBS-Tween with 5% BSA. Membranes were washed with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-Tween) four times for 15 minutes at a time. Membranes were then incubated with the relevant secondary antibody (Table 2-2) diluted in TBS-Tween and 5% BSA for 1 hour at room temperature. Membranes were then washed again four times with TBS-Tween for 15 minutes at a time followed by a final wash for 15 minutes with TBS. Signals were detected using the Amersham ECL detection system (GE Healthcare Europe GmBH, Munich, Germany) as described. Strength of western blots was measured using Scion image for windows software (Scion Corporation) and standardised to basal levels against a reference gene where appropriate.

Primary antibody	Concentration	Secondary antibody	Concentration
Chemerin	1 : 1000	Anti – goat	1: 2000
ChemR23	1:1000	Anti – goat	1 : 2000
Phospho – AKT	1 : 2000	Anti – rabbit	1 : 10 000
Total – AKT	1 : 2000	Anti – rabbit	1 : 2000
Phospho – ERK ½	1 : 2000	Anti – mouse	1 : 10 000
Total – ERK 1/2	1 : 2000	Anti – rabbit	1 : 2000
Phospho – 4EBP1	1 : 1000	Anti – rabbit	1: 5000
Phospho – S6 Kinase	1 : 1000	Anti – rabbit	1 : 5000
AGR2	1: 2000	Anti – sheep	1 : 4000
GAPDH	1 : 25 000	Anti – mouse	1 : 10 000
MMP 2	1: 200	Anti – mouse	1 : 5000

Table 2-2: Table demonstrating primary antibodies used for WBA with concentrations and relevent secondary antibodies.

2.5 Cell proliferation assay

Cell proliferation assays were conducted using PC3 and LNCaP cells. Cells were uniformly seeded onto 96-well plates and were incubated with serum-free media for 16 hours prior to being treated for 24 hours with the following concentrations of chemerin supplemented to the media: 0nM (Basal), 0.1nM, 1nM and 10nM chemerin (R & D systems). Media supplemented with 10% FCS was used as a positive control (not shown for PC3 cells). Cell proliferation was assayed using a CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI, USA). The homogeneous "add-mix-measure" format results in cell lysis and generation of a luminescent signal proportional to the amount of adenosine triphosphate (ATP) present and uses a luciferase reaction. The amount of ATP is directly proportional to the number of cells present in culture in agreement with previous reports.

Luminescence was measured by absorbance at 490 nm using a spectrophotometric plate reader (TECAN, Männedorf, Switzerland).

2.6 Cell invasion assay

The BD BioCoat™ Tumour invasion system (BD, Bedford, MA, USA) was used for the cell invasion assay. The system comprises of a BD Falcon™ FluoroBlock™ 96-Multiwell Insert Plate with an 8.0 micron pore size Polyethylene terephthalate (PET) membrane that is uniformly coated with BD Matrigel™ Matrix. This matrix serves as a reconstituted basement membrane *in vitro* providing a true barrier to non-invasive cells while presenting an appropriate protein structure to study invasion. 25µl of cell suspension (1.25×10^4 cells and 25µl of serum-free media was added to each of the apical chambers after rehydration of the membrane. Using the sample ports for access 200µl serum-free media containing the following treatments were added to the basal chambers: Basal (0nM), 0.1nM, 1nM and 10nM chemerin and FCS (10%). Cells were post-labelled by placing the insert system into a second 96-well plate containing 200µl/well of 4µg/ml calcein AM in serum-free media for 1 hour at 37^{o}C, 5% CO_2. Fluorescence of invaded cells were detected by a bottom-reading plate reader at wavelengths of 494/517nM (Ex/Em).

2.7 Wound Healing (Migration) assay

PC3 and LNCaP Cells were cultured as described earlier and were grown to 100% confluence on 6 well plates. Cells were incubated in serum-free media for 24 hours prior to treatment with increasing doses of chemerin (0-10nM). A scratch or "wound" was applied to well by drawing a 200µl pipette tip vertically down the well. Pictures of the wound closure were taken at 24 hours on a microscope and camera. Three pictures from each scratch were taken and analysed. ImageJ software (public domain) was used to measure closure of the "wound".

2.8 Apoptosis assay

PC3 and LNCaP cells were cultured as before and seeded onto 24-well plates for the apoptosis assy. Cells were incubated in serum-free media for at least 4h prior to commencement of cell treatments with 0nM (basal), 0.1nM, 1nM and 10nM chemerin as well as a positive control: Hydrogen peroxide (1:200 000). After the respective time period had elapsed cells *all* cells were labelled with Hoechst® stain (Invitrogen, Eugene, OR, USA) for 15 minutes prior to the addition of the nucleic acid stain YO-PRO® (Life Technologies, Carlsbad, CA, USA). Three separate time-points were used for the apoptosis assay: 4, 12 and 24 hours. YO-PRO® is an iodine-based stain that *only* labels cells that are undergoing apoptosis and does not label living cells, therefore allowing detection of cells undergoing apoptosis using fluorescent microscopy (57).YO-PRO® allows assessment of apoptosis without interfering with cell viability. Cells were labelled for a further 30 minutes with YO-PRO® and were washed twice afterwards with warmed PBS. Positive controls were measured after 4 hours as this was the time at which maximal apoptosis was to be expected. Plates were imaged using the GE healthcare IN Cell Analyzer 1000. Number of apoptotic cells was calculated using the formula below:

$$\frac{\text{YO-PRO® - labelled (apoptotic) cells}}{\text{Hoechst® - labelled (total) cells}} \times 100 = \textbf{\% rate of apoptosis}$$

2.9 Enzyme-Linked Immunosorbent Assay (ELISA)

Serum chemerin levels were calculated using a sandwich ELISA kit (DuoSet ELISA Development kit, R & D Systems, Abingdon, UK) in a 96-well plate. Firstly, the assay was optimised using the chemerin standard provided diluted in reagent diluent (1% BSA in PBS 0.2µm filtered) to create a seven point standard curve using 2-fold serial dilutions. A high standard of 2000 pg/mL was used as recommended by the protocol. Serum samples were diluted 8-fold in reagent diluent in order to obtain the most accurate chemerin level against the standard curve. Capture antibody was diluted to the working concentration and 100µl

was added to each well of the plate which was sealed and incubated at room temperature overnight. The capture antibody was removed and each well was washed three times with 400 µl of wash buffer (0.05% Tween® 20 in PBS). The plate was blotted dry after each subsequent wash with clean paper towels. The plate was blocked by adding 300µl/well of reagent diluent and incubating at room temperature for 1 hour. The reagent diluent was removed and each well was washed again three times with wash buffer. 100µl of the pre-diluted serum samples was added to each well and the plate was covered and incubated at room temperature for a further 2 hours. Following removal of the samples and three more washes, 100µl of the detection antibody diluted to the working concentration in reagent diluent was added to each well. The plate was covered with a fresh adhesive strip and incubated at room temperature for a further 2 hours. 100µl of the working concentration of streptavidin conjugated to horseradish-peroxidase (HRP) was added to each well following a further cycle of aspiration /washes. The plate was covered and incubated at room temperature for 20 minutes away from direct light. The streptavidin-HRP solution was aspirated from each well and each well was washed three times as before with wash buffer. 100µl of substrate solution (1:1 mixture of H_2O_2 and Tetramethylbenzidine) was then added to each well, before the well was covered and incubated away from direct light for a further 20 minutes. After 20 minutes 50µl of stop solution ($2NH_2SO4$) was added to each well and the plate was gently tapped to ensure thorough mixing of the solutions. The absorbance of each well was determined immediately using a micro-plate reader (Tecan Group Ltd. Männedorf, Switzerland) and Magellan™ software at 450nm with wavelength correction set at 540nm.

2.10 Methods for assessing the effects of chemerin on signal transduction pathways

In order to assess the role that chemerin plays in AKT, ERK and mTOR activation PC3 and LNCaP cells were cultured as previously described until 80% confluent and seeded on to 6 well plates. Cells were incubated with starvation media containing 1nM chemerin for the

following time-points (minutes): 0 (basal), 2, 5, 15, 30 and 60. Insulin (AKT & ERK 1/2 – 5 min) and phorbol 12-myristate 13-acetate (PMA) (mTOR activation - 30 min) were used as positive controls as they are known to activate the AKT (58), ERK 1/2 (59) and mTOR (60) pathways respectively at these relative time points. Once the appropriate time had elapsed 1ml of ice-cold PBS was added to each well to arrest cell growth. Cells were lysed with RIPA buffer as before and the subsequent protein lysate was extracted and immediately frozen on dry ice. Protein lysates were quantified using the BCA method and diluted as necessary using 2x samples buffer, Laemmli (Sigma Aldrich) to ensure standardisation. WBA was carried out using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described using the antibodies according to the table in the materials and methods section (see Table 2-2).

2.11 Methods for assessing the effects of chemerin on AGR2 expression

The prostate cancer cell lines PC3 and LNCaP were cultured as before until confluent in 6 well plates. Cells were incubated in serum starvation media for at least 16 hours (overnight) prior to treatments with chemerin. AGR2 protein and mRNA expression studies were conducted using varying concentrations of recombinant human chemerin (R & D systems) varying from 0 (basal) to 10nM supplemented to the media and the cells were incubated for varying time points (4 – 36 h). In order to evaluate the pathways by which chemerin ultimately exerts its effects on AGR2 expression, varying cancer pathway inhibitors were used to treat the PCa cells (PC3 cells only). Cells were treated for 36 hours and the resultant protein lysates were quantified as before and then analysed using WBA described below using the AGR2 primary antibody. The inhibitors included: ERK, AKT, PKA, PKC and mTOR inhibitors (Figure 2-1 . The inhibitors used are named in Table 2-3 and were utilised at the concentrations demonstrated for the appropriate pre-incubation period.

Target protein	Name	Pre-incubation (minutes)	Concentration
ERK1/2	UO126	60	10 µM
AKT	Wortmannin	60	100 nM
PKA	H-89	30	10 µM
PKC	Bis(indolyl)maleimide	120	1 µM
mTOR	Pp242	60	100 nM

Table 2-3: Table demonstrating relevant protein inhibitor, name, time for pre-incubation (minutes) and concentration used.

mRNA expression was evaluated using the qRT-PCR method described earlier. AGR2 (Integrated Design Technologies) and GAPDH (PrimerDesign) sense and antisense primers used were as described (Table 2-1). Experiments were performed in triplicate and results shown are expressed as the mean (+ SEM). AGR2 protein expression was demonstrated using WBA using an AGR2 anti-Sheep primary antibody (R & D systems) at the appropriate dilution and a secondary anti-sheep antibody (see Table 2-2). Blots were also probed for the reference gene GAPDH using a GAPDH anti-mouse primary antibody (see Table 2-2) and an anti-mouse secondary antibody to ensure equal protein loading.

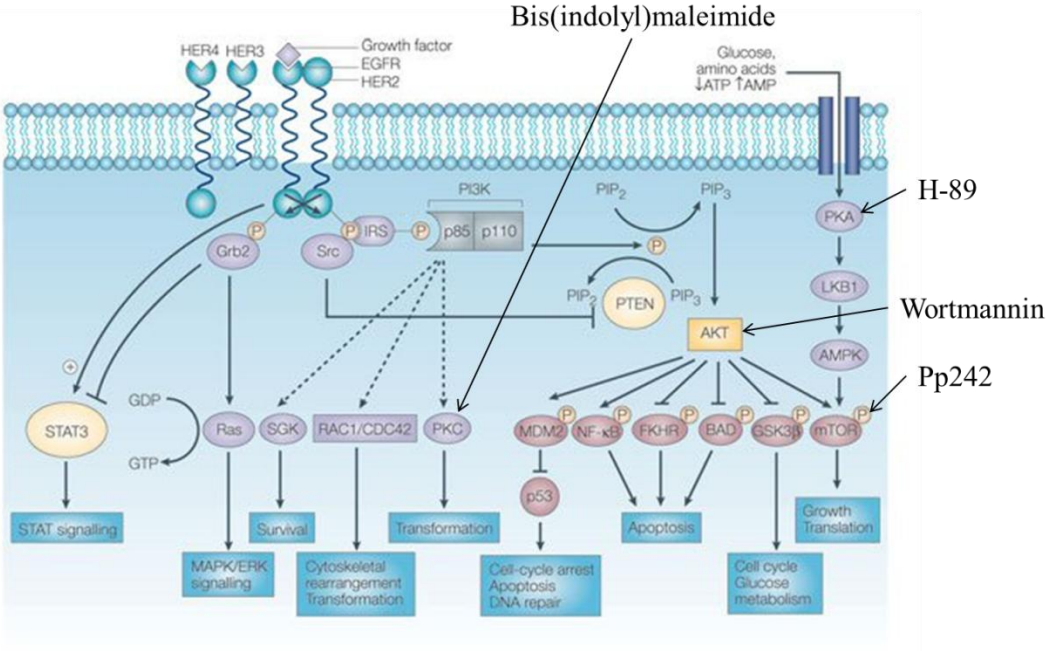

Figure 2-1: Diagram demonstrating site of targets for inhibitors in AKT pathway used during experiments (61).

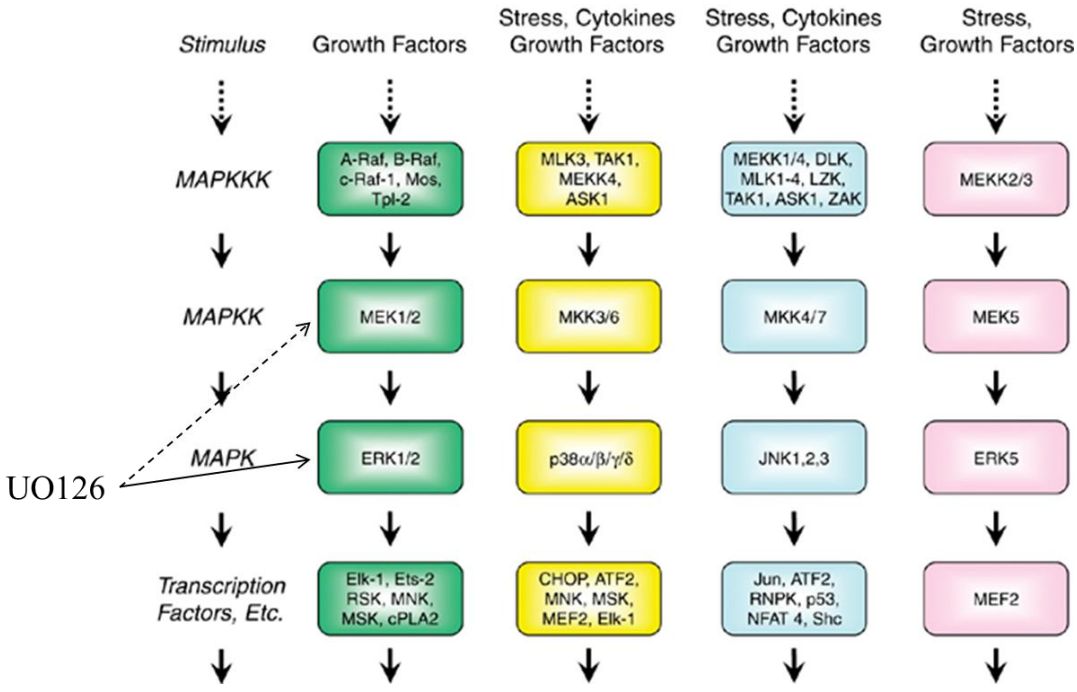

Figure 2-2: Diagram demonstrating site of targets for inhibitors in ERK pathway used during experiments (62).

2.12 Statistical analysis

Comparison of means of multiple groups was performed using GraphPad Prism statistics software (GraphPad Software, Inc.) Means were compared using the one-way ANOVA method: Tukey's post hoc analysis. Experiments were conducted in triplicate unless stated otherwise and results are demonstrated as a bar chart where applicable with the mean and standard error of the mean (+ SEM) demonstrated.

3 The expression of chemerin and its receptor (ChemR23) in prostate cancer cells and human prostate tissue

3.1 Collection of human samples

Human prostate tissue was obtained from men undergoing prostate procedures (e.g. radical retro-pubic prostatectomy (RRP), transurethral resection of the prostate (TURP) and trans-rectal ultrasound (TRUS) and prostate biopsy) at University Hospital, Coventry (University hospitals Coventry & Warwickshire (UHCW) NHS Trust). A favourable ethical opinion was obtained from the NHS National Research Ethics Service (North West 3 Research Ethics Committee - Liverpool East) prior to tissue collection. Local Research & Development (UHCW NHS Trust) approval was also obtained prior to the commencement of tissue collection at University Hospital, Coventry. Men undergoing prostate surgery for either benign or malignant conditions or undergoing a prostate biopsy for suspected cancer (either with an elevated age-specific PSA or with an abnormal prostate examination) were approached for possible inclusion into the study. Patients were given a full explanation of the nature of the study including the potential benefits and risks of taking part. Potential participants were also given an information sheet (see Appendix A) with which to make an informed decision about their inclusion in the study. Patients who were willing to be recruited for inclusion in the study were asked to sign a consent form. Patients received a signed copy of the consent form (see Appendix B) and a further signed copy of the consent form was subsequently filed in the patient records. In some cases patients had catheters in situ and so this was noted at the time of recruitment. The patients' medical and drug history was taken as well as any prior treatment, if any, for PCa. During TURP procedures and TRUS & prostate biopsies prostate tissue was collected and immediately snap frozen in liquid nitrogen and stored at -80^{o}C until use. Radical prostatectomy specimens were removed en-bloc and were formalin-fixed-paraffin-embedded as per standard hospital

practice. Tissue was available for use after the hospital pathologist had issued a final pathology report of the specimen for grading/staging purposes.

3.2 Chemerin and ChemR23 mRNA expression in human prostate tissue and cell lines

The Human prostate cancer cell lines PC3 and LNCaP were used to demonstrate the presence of the DNA of both chemerin peptide and its receptor: ChemR23. PCa cells were cultured and the extracted RNA was converted into cDNA according to the method previously described. The cDNA was amplified using RT PCR and subsequent agarose gel electrophoresis was performed on the PCR products in order to demonstrate the presence of chemerin and ChemR23 DNA. The following samples were used: benign human prostate tissue, PC3 cells, LNCaP cells, positive control (human adipocyte cells) and negative control (H_20).

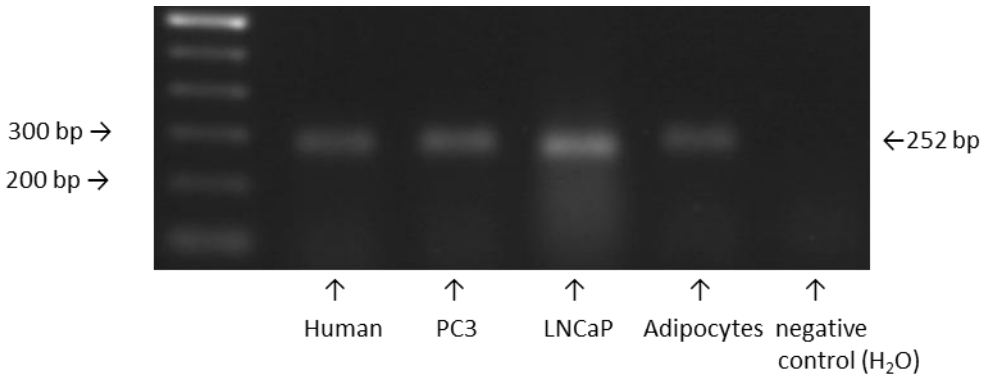

Figure 3-1: Representative agarose gel demonstrating expression of chemerin cDNA in human prostate tissue and prostate cancer cell lines.

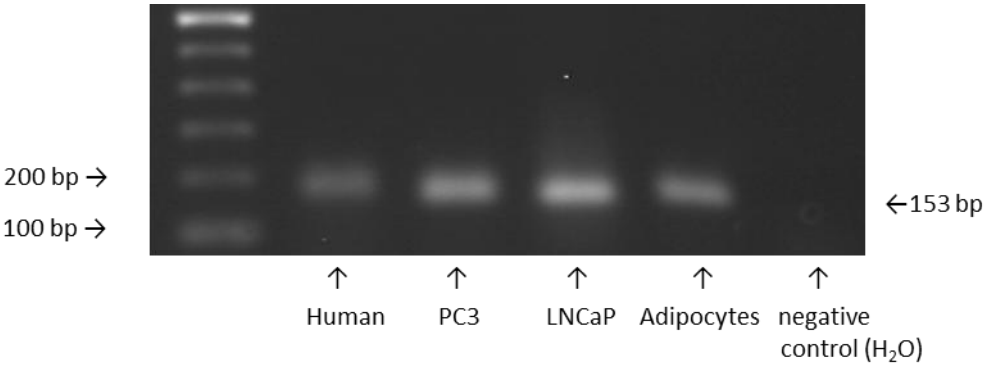

Figure 3-2: Representative agarose gel demonstrating expression of ChemR23 cDNA in human prostate tissue and prostate cancer cell lines.

In order to assess whether there was any difference between the expression of chemerin/ChemR23 mRNA between benign and malignant prostate tissue five fresh prostate samples were identified from each group (benign & malignant). The quantity of mRNA expression in each sample was determined by qRT-PCR. The reference gene GAPDH was used as an internal control for each sample. The following graph demonstrates the difference in expression between the two groups for chemerin/ChemR23 mRNA (n = 5).

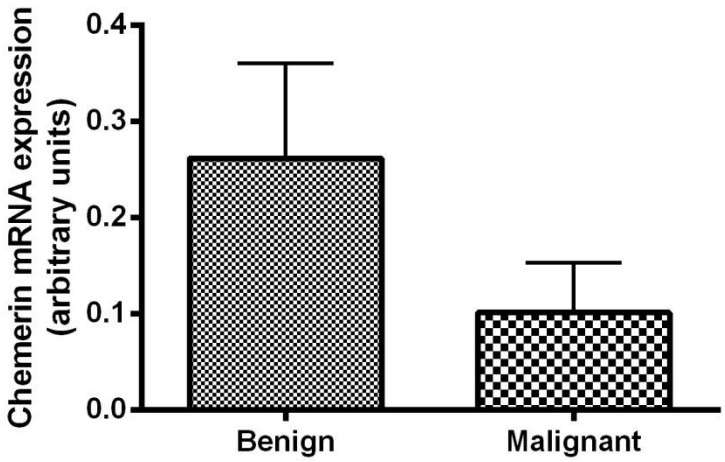

Figure 3-3: Bar chart demonstrating chemerin mRNA expression in benign and malignant prostate tissue, (p = 0.1904, for n = 5, mean + SEM).

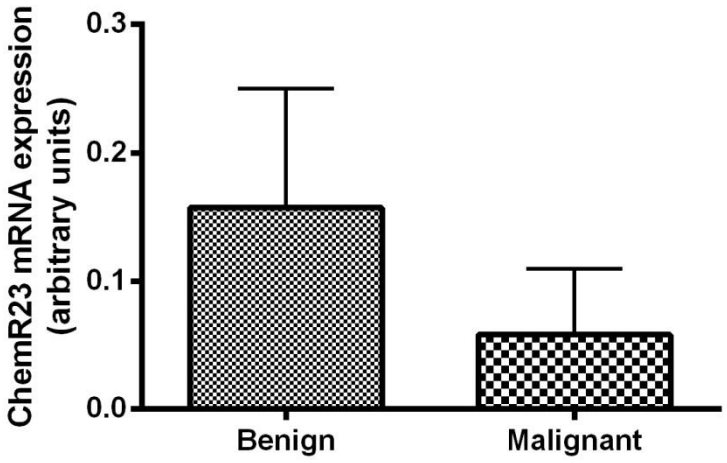

Figure 3-4: Bar chart demonstrating ChemR23 mRNA expression in benign and malignant prostate tissue, (p = 0.3785, for n = 5, mean + SEM).

Although there appeared to be an increase in both chemerin and ChemR23 Protein expression in benign tissue with respect to malignant this was not statistically significant (according to a t-test) for chemerin (p = 0.1904) or ChemR23 (p = 0.3785). The reasons for this are potentially multiple and will be discussed later.

3.3 Chemerin and ChemR23 protein expression in human prostate tissue and cell lines

Protein was extracted from benign human prostate tissue, PC3 and LNCaP cells and human adipocyte cells in order to demonstrate the presence of chemerin and ChemR23 protein. Protein levels were demonstrated using WBA using the relevant antibodies Anti-chemerin & anti-ChemR23 (Santa Cruz biotechnology, Middlesex, UK) with secondary anti-goat (Dako Ltd., Cambridge, UK). Please see Table 2-2 for the relevant antibody concentrations.

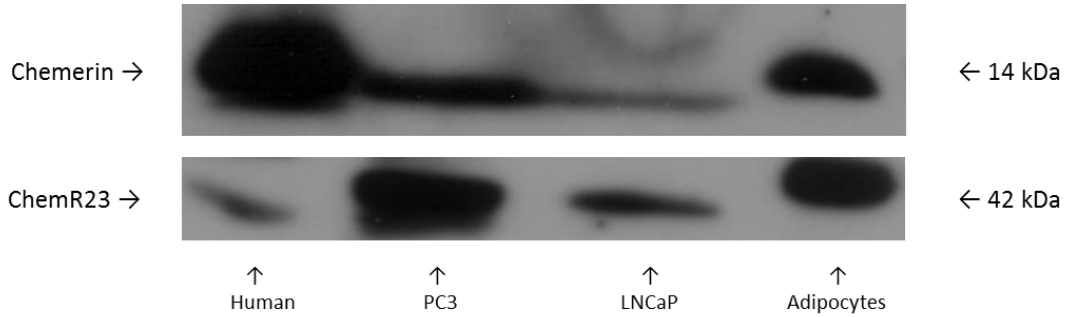

Figure 3-5: Representative blot demonstrating expression of ChemR23 protein in human prostate tissue and prostate cancer cell lines.

Although the apparent expression of chemerin and its receptor: ChemR23 varies greatly between human prostate tissue, prostate cancer and the adipocyte cell line few conclusions can be drawn about significant differences as the protein concentration in each respective lysate varies also. The purpose of this western blot is simply to demonstrate the presence of chemerin and its receptor (ChemR23) in prostate cell lines and human prostate tissue.

As with mRNA in order to demonstrate any difference between chemerin and ChemR23 protein expression in benign and malignant four samples from each group (benign & malignant) were used. The resultant western blot is demonstrated below:

Figure 3-6: Representative western blot demonstrating chemerin/ChemR23 protein expression in benign and malignant human prostate tissue.

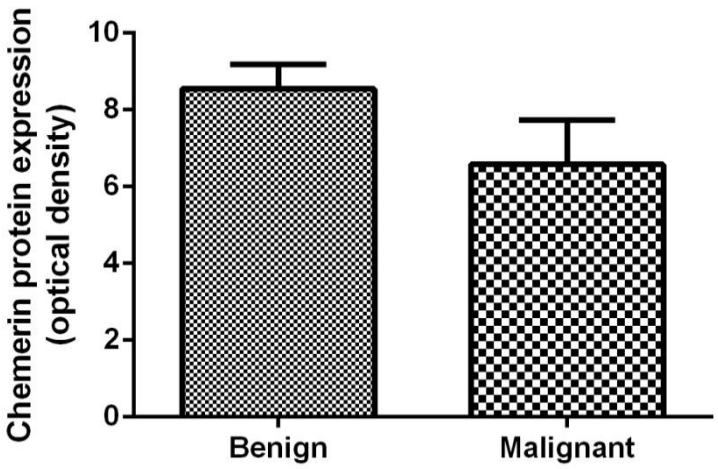

Figure 3-7: Bar chart demonstrating chemerin protein expression in benign and malignant prostate tissue, (p = 0.1893 for n = 4, mean + SEM).

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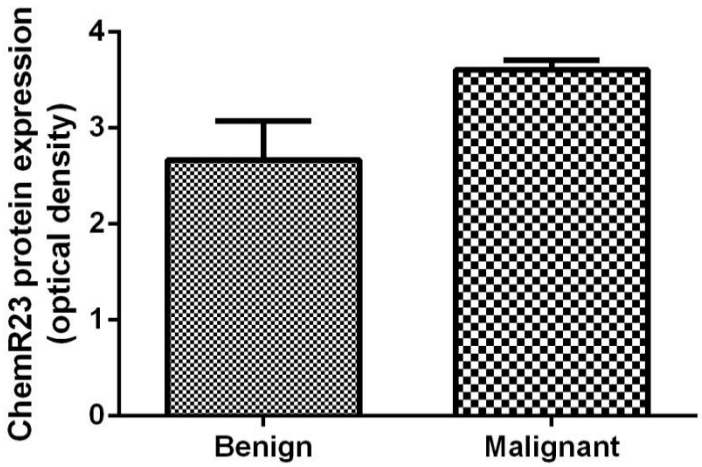


Figure 3-8: Bar chart demonstrating ChemR23 protein expression in benign and malignant prostate tissue, (p= 0.0657 for n = 4, mean + SEM).

Quantification of the western blot was performed and the resultant graphs are shown above. As with mRNA, expression of chemerin protein in benign tissue appeared to be higher compared to malignant tissue although this result was not statistically significant (p = 0.1893). ChemR23 protein expression appeared to be higher in malignant prostate tissue compared with benign although again this was not statistically significant (p = 0.0657).

3.4 Discussion

Demonstrating the presence of the adipokine peptide, chemerin and its receptor (ChemR23) mRNA and protein in human prostate tissue and cells is fundamental to the hypothesis that chemerin potentially, has a role to play in PCa, and in particular obesity-induced prostate cancer. As far as this author is aware this is the first time that chemerin and ChemR23 mRNA and protein have been demonstrated to be present in prostate tissue and the PCa cell lines: PC3 and LNCaP. Due to the varying concentrations of mRNA found in prostate cancer cells and human prostate tissue in the samples no direct comparisons can be drawn between the rates of expression. The purpose of the electrophoresis of the PCR products is simply to demonstrate that chemerin (Figure 3-1) and ChemR23 (Figure 3-2) mRNA is present. The rates of chemerin and ChemR23 mRNA expression were also analysed comparing benign with malignant prostate tissue (Figure 3-3 & Figure 3-4). The increased expression of chemerin and ChemR23 mRNA found in benign prostate tissue was not statistically significant. Only 5 samples were used for this experiment and were not corrected for factors such as age, grade of cancer and drug treatment. The differences therefore seen cannot be attributed solely to whether the tissue was benign or malignant. Larger numbers of more closely matched patients in terms of age, stage/grade of prostate cancer and drug treatment would be needed in order to accurately determine whether there is a difference in expression of chemerin and ChemR23 mRNA between benign and malignant prostate tissue. It could be possible that the higher levels of chemerin/ChemR23 found in benign prostate tissue leads to a more optimum cellular microenvironment within the prostate gland permitting and promoting the development of prostate cancer. When prostate tissue becomes cancerous and therefore less similar to its native tissue it becomes poorly differentiated and therefore the apparent *normal* production of chemerin/ChemR23 mRNA may be lost. Figure 3-5 demonstrates the presence of chemerin/ChemR23 protein in human prostate tissue as well as the human prostate cancer cell lines: PC3 and LNCaP. Human adipocyte cells were used as a positive control as these cells are known to express chemerin/ChemR23. Figure 3-7 &

Figure 3-8 demonstrate the levels of chemerin/ChemR23 protein according to the western blot data (Figure 3-6). As with chemerin mRNA, levels of chemerin protein expression appeared to be *higher* in benign tissue compared with malignant tissue albeit not at a significant level. ChemR23 protein expression was slightly higher in malignant tissue according to the western blot data. The reasons for the difference between ChemR23 mRNA and protein expression in malignant tissue possibly reflects the heterogeneous nature of malignant tissue and indeed the differences between the patients in terms of age, stage/grade of cancer and treatment received.

4 The effect of chemerin on cell proliferation, invasion, migration and apoptosis in prostate cancer cell lines

4.1 Cell proliferation assay

Cell culture was performed as per the earlier protocol and the experiments were performed in quadruplicate. Results are demonstrated as the mean + SEM and are relative to basal after 24 hours treatment.

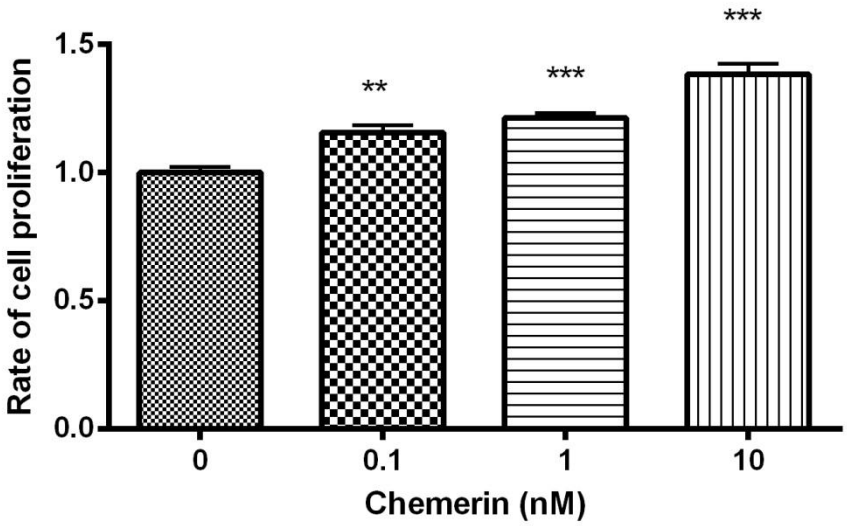


Figure 4-1: Bar chart demonstrating the effects of chemerin on cell proliferation in PC3 cells (n = 4) after 24 hours with respect to basal, (mean + SEM, difference from basal, **: p<0.01, ***: p<0.001).

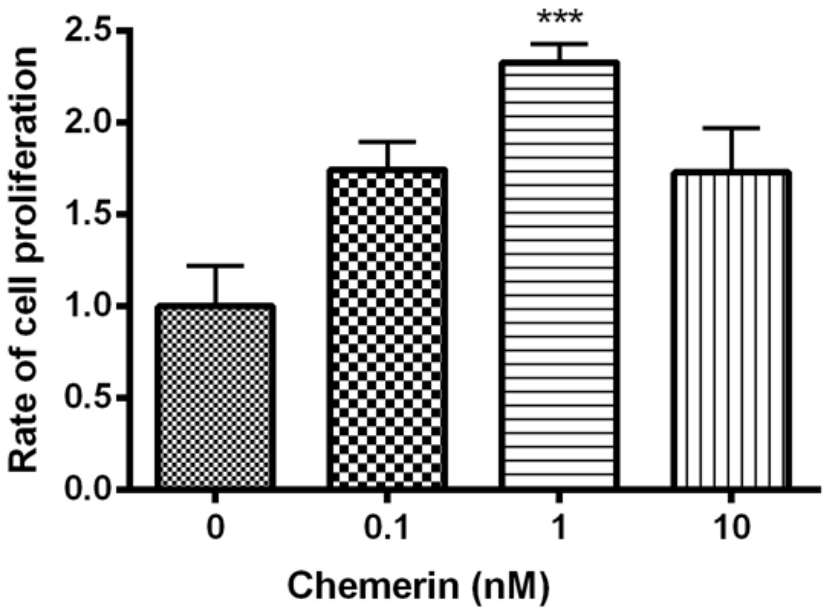


Figure 4-2: Bar chart demonstrating the effects of chemerin on cell proliferation in LNCaP cells (n = 4) after 24 hours with respect to basal, (mean + SEM, difference from basal, ***: p<0.001).

In PC3 cells there was a significant increase in cell proliferation with increasing levels of chemerin after 24 hours treatment. For 0.1nM, 1nM and 10nM chemerin the statistical significance difference between cell proliferation and basal (0nM) was $p < 0.01$(0.1nM) and $p < 0.001$ for 1nM & 10nM chemerin. In LNCaP cells there was a much larger increase in cell proliferation with chemerin after 24 hours treatment compared with PC3 cells (~2-fold vs ~1.3-fold). The only statistically significant difference in cell proliferation was noted between 1nM chemerin and basal (0nM) ($p < 0.001$) after 24 hours. 10% FCS was used as a positive control and also lead to a statistically significant increase ($p < 0.05$) in cell proliferation after 24hours.

4.2 Cell invasion assay

The cell invasion assay was carried out as described earlier as per the manufacturer's protocol. Results are demonstrated as mean + SEM and were performed in triplicate. Results relative to basal are shown after 24 hours incubation.

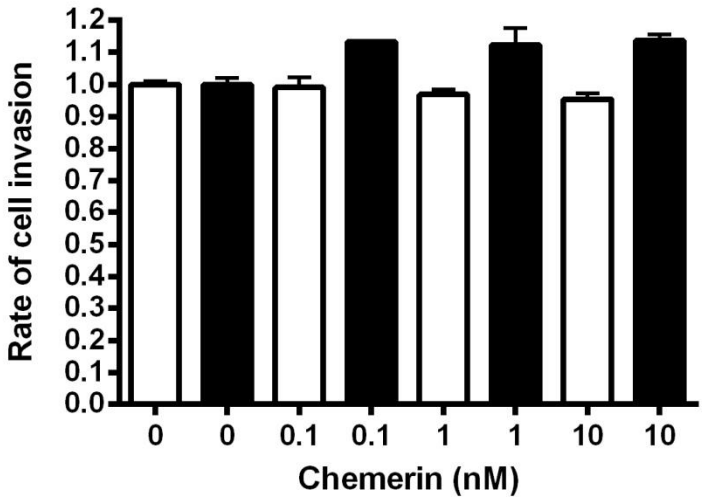


Figure 4-3: Bar chart demonstrating the effects of chemerin on invasion of PC3 (white) and LNCaP (black) cells (n = 3) after 24 hours.

There appeared to a slight increase in cell invasion in LNCaP cells at all concentrations of chemerin however this was not statistically significant. There was no difference in cell invasion in PC3 cells.

4.3 Wound Healing (Migration) assay

A wound healing assay was carried out as per the earlier protocol described and the experiments were performed in triplicate (see Figure 4-4 & Figure 4-5). The results demonstrated are presented as the mean + SEM after 24 hours incubation.

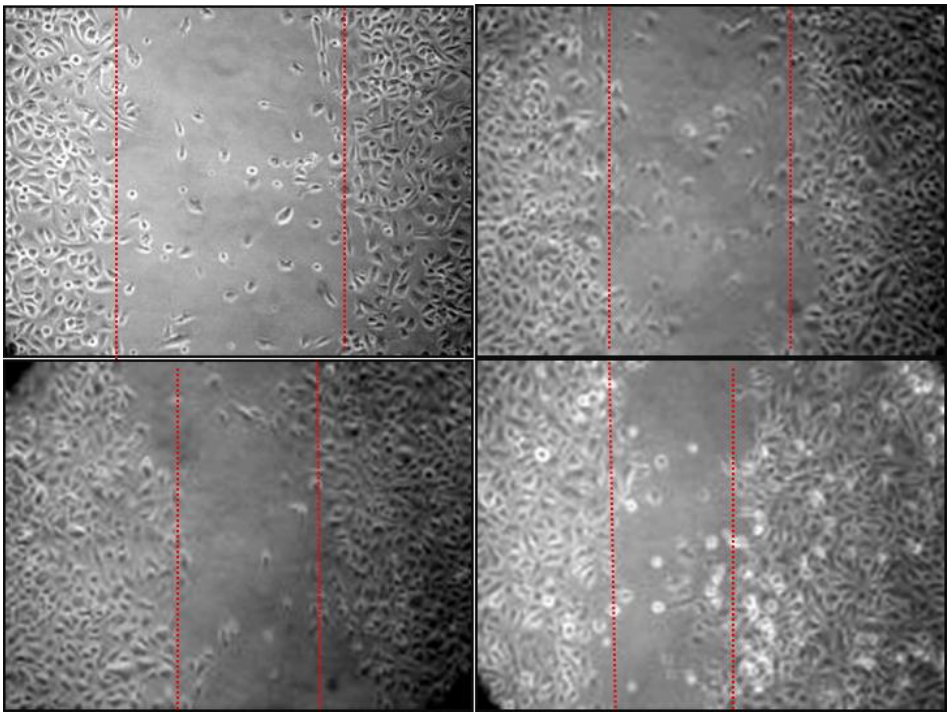


Figure 4-4: Photos of migration distances ("wound" closure) for PC3 Cells for: (clockwise from top left) Basal (0nM chemerin), 1nM chemerin, 10%FCS (positive control) and 10nM chemerin respectively.

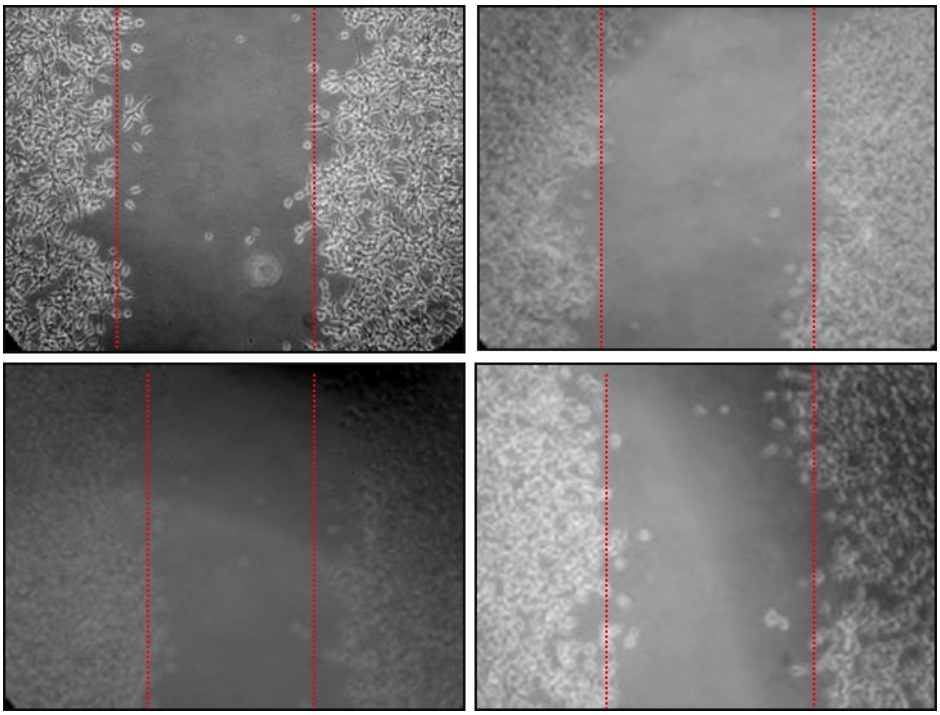


Figure 4-5: Photos of migration distances ("wound" closure) for LNCaP Cells for: (clockwise from top left) Basal (0nM chemerin), 1nM chemerin, 100nM DHT (positive control) and 10nM chemerin respectively.

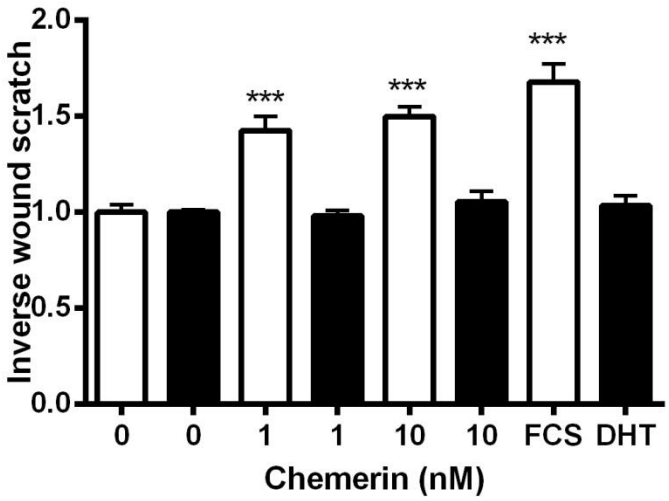


Figure 4-6: Bar chart demonstrating the effects of chemerin on migration of PC3 (white) and LNCaP cells (black) after 24 hours (n = 3), (mean + SEM, difference from basal, ***: $p < 0.001$).

In the wound scratch assay there was again a significant increase in the inverse of the wound scratch distance in PC3 cells. Because cells migrate into the "wound" with time, the more they migrate, the *lower* the distance at the end of the experiment between the cells. Therefore the *higher* the inverse of the wound scratch distance the higher the degree of migration. 1nM, 10nM chemerin and 10%FCS were all significantly associated with increased migration ($p < 0.001$) with respect to basal. No significant effect was demonstrated in the LNCaP cells after 24 hours.

4.4 Apoptosis assay

The apoptosis assay was carried out as per the protocol described earlier for 3 time points (4, 12 and 24 hours). Results are demonstrated as mean + SEM and were performed in triplicate.

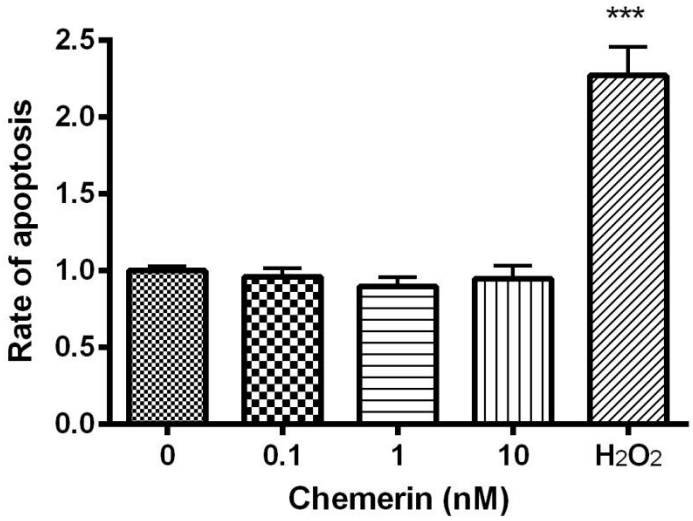


Figure 4-7: Bar chart demonstrating effects of chemerin on apoptosis of PC3 cells (n = 3) after 4 hours, (mean + SEM, difference from basal, ***: p < 0.001).

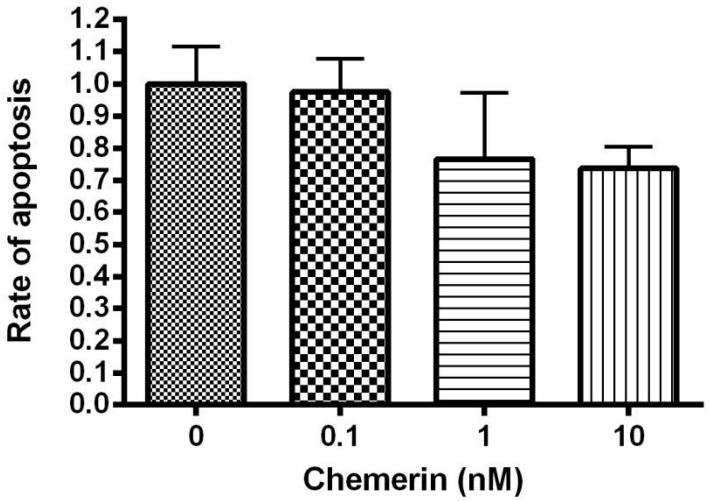


Figure 4-8: Bar chart demonstrating effects of chemerin on apoptosis of PC3 cells (n = 3) after 12 hours, (mean + SEM).

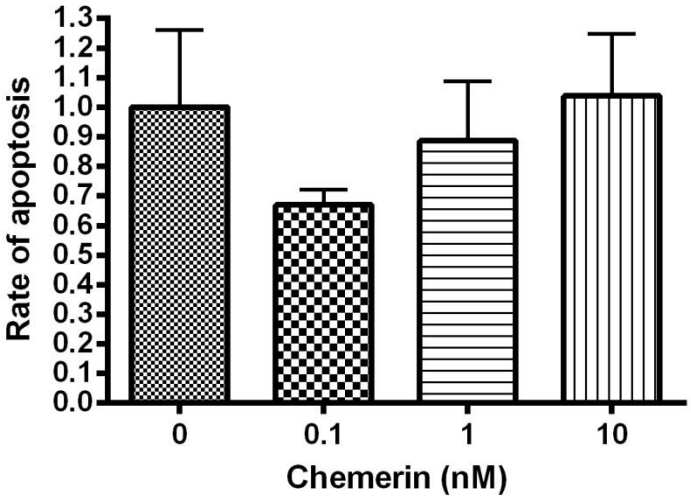


Figure 4-9: Bar chart demonstrating effects of chemerin on apoptosis of PC3 cells (n = 3) after 12 hours, (mean + SEM).

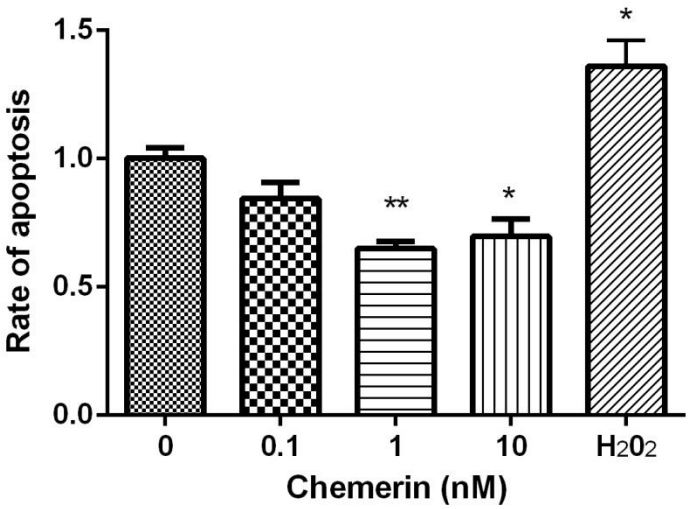


Figure 4-10: Bar chart demonstrating effects of chemerin on apoptosis of LNCaP cells (n = 3) after 4 hours, (mean + SEM, difference from basal, *: $p < 0.05$, **: $p < 0.01$).

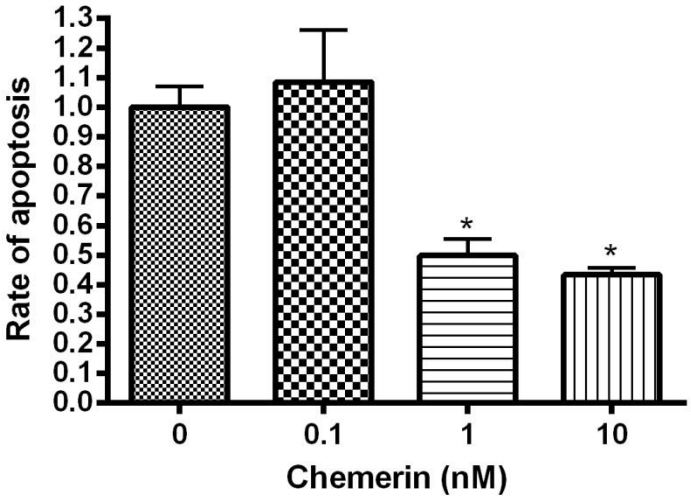


Figure 4-11: Bar chart demonstrating effects of chemerin on apoptosis of LNCaP cells (n = 3), after 12 hours (mean + SEM, difference from basal, *: p < 0.05).

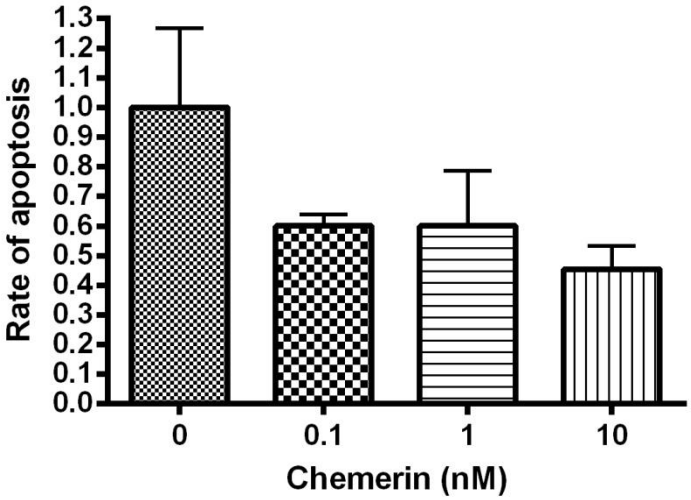


Figure 4-12: Bar chart demonstrating effects of chemerin on apoptosis of LNCaP cells (n = 3) after 24 hours, (mean + SEM).

Hydrogen peroxide (H_2O_2) was used as a positive control and showed a significant increase in apoptosis of PC3 cells ($p < 0.001$) and LNCaP cells ($p < 0.05$) after 4 hours. Hydrogen peroxide was only used for a short time point (4hr) as beyond this time point cells were likely to be in a state of secondary necrosis and thus undetectable as apoptotic using the YO-PRO® method. With regards to PC3 cells there appeared to be a slight reduction in apoptosis

at the higher doses of chemerin (1nM & 10nM) after 12 hours stimulation; however this was not statistically significant. There also appeared to be a decrease in apoptosis of PC3 cells with 0.1nM chemerin after 24 hours however again this was not statistically significant. In the LNCaP cell line there appeared to be decrease in apoptosis at all concentrations of chemerin (0.1-10nM) at all time points (4 – 24Hr) apart from 0.1nM chemerin after 12 hours relative to basal.

				Time (Hours)		
				4	12	24
Cell line	PC3	**Chemerin treatment (nM)**	H_2O_2	+++		
			0.1	+/-	+/-	+/-
			1	+/-	+/-	+/-
			10	+/-	+/-	+/-
	LNCaP		H_2O_2	+		
			0.1	+/-	+/-	+/-
			1	--	-	+/-
			10	-	-	+/-

Figure 4-13: Table demonstrating the overall effects of chemerin on cellular proliferation in PC3 and LNCaP cells. Level of significance of increase/decrease of apoptosis: +/- = ($p < 0.05$), ++/-- = ($p < 0.01$) and +++/--- = ($p < 0.001$).

4.5 The effect of chemerin on key intracellular regulatory mechanisms in prostate cancer cells.

In order to examine what the underlying pathways are by which these cellular changes are brought about, RNA was extracted from PC3 and LNCaP cells after treatment with chemerin for 4 and 24 hours as previously described. The RNA was converted into cDNA and primers for the following genes were used: p53, B-cell lymphoma 2 **(**bcl-2), Bcl-2-associated X protein (Bax), and Bcl-2-like protein 1 (bcl-2 L1) to amplify the resultant quantity of cDNA

(Table 2-1). These genes are commonly implicated in prostate carcinogenesis (see 4.6) and so differing expressions of these genes was examined in PCa cells. The resultant mRNA levels using primers for GAPDH as an internal control are demonstrated below:

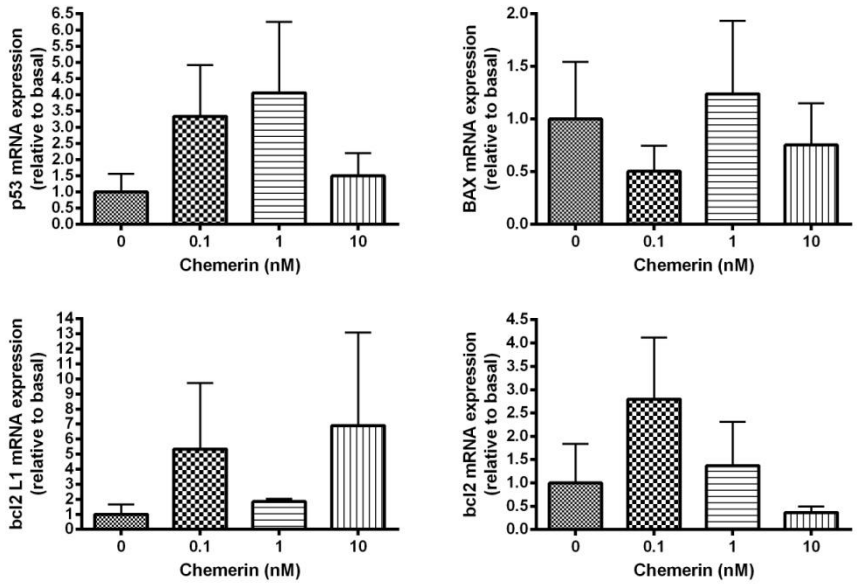


Figure 4-14: Bar charts demonstrating the effects of chemerin on (clockwise) p53, BAX, bcl2 and bcl2 L1 expression in PC3 cells (n = 3) with respect to basal after 4 hours, (mean + SEM).

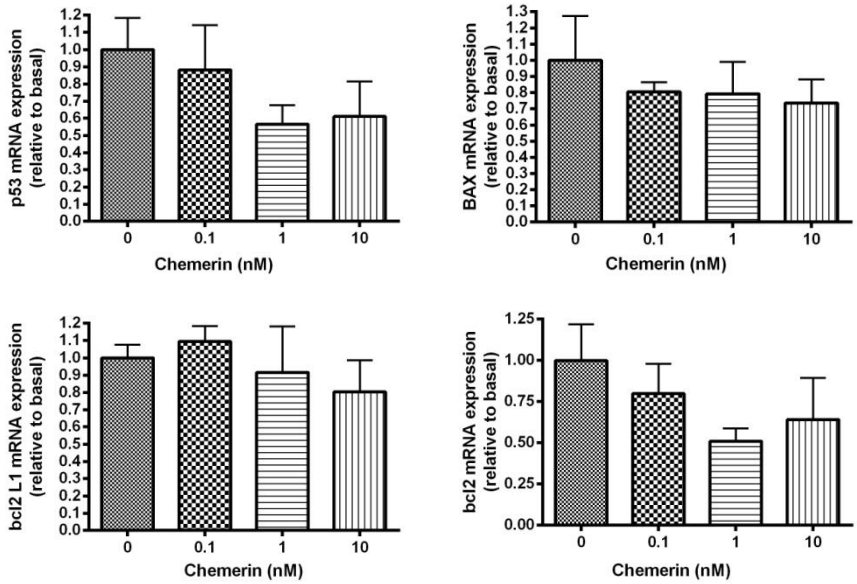


Figure 4-15: Bar charts demonstrating the effects of chemerin on (clockwise) p53, BAX, bcl2 and bcl2 L1 expression in LNCaP cells (n = 3) with respect to basal after 4 hours, (mean + SEM).

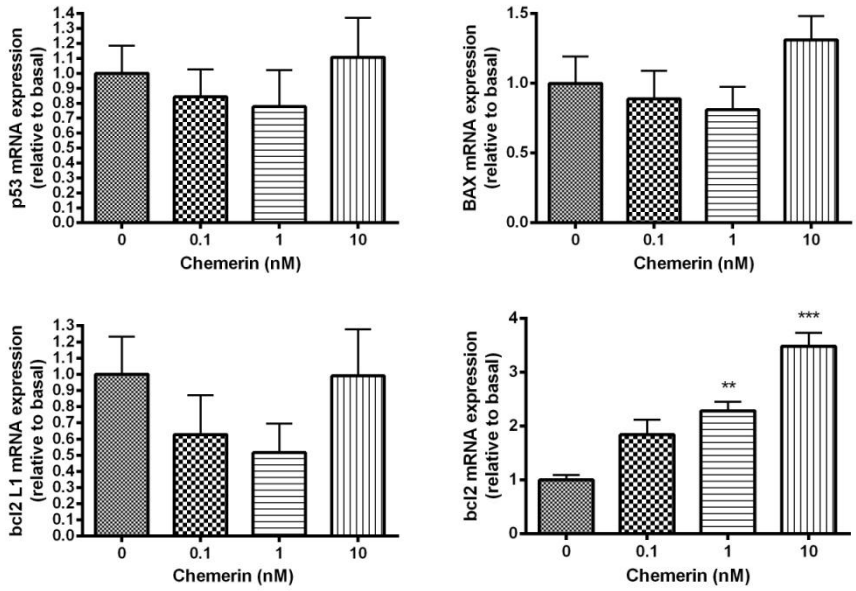


Figure 4-16: Bar charts demonstrating the effects of chemerin on (clockwise) p53, BAX, bcl2 and bcl2 L1 expression in PC3 cells (n = 3) with respect to basal after 24 hours, (mean + SEM, difference from basal, **: p<0.01, ***: p<0.001).

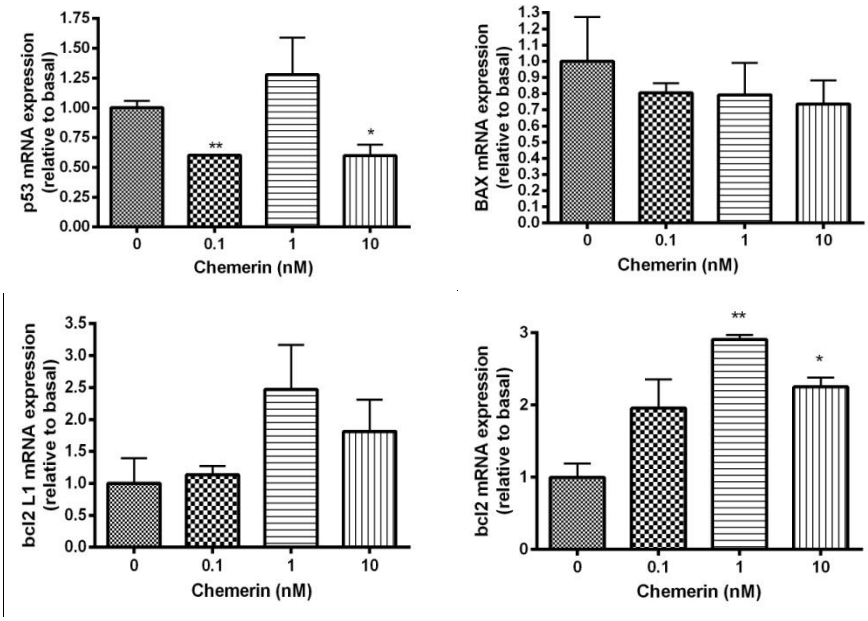


Figure 4-17: Bar charts demonstrating the effects of chemerin on (clockwise) p53, BAX, bcl2 and bcl2 L1 expression in LNCaP cells (n = 3) with respect to basal after 24 hours (mean + SEM, difference from basal, *: p < 0.05, **: p < 0.01).

After 4 hours chemerin treatment no statistically significant changes were seen in the mRNA expression of p53, Bax, bcl-2 or bcl-2 L1 in either cell line. There appeared to be a *reduction* in p53 and bcl-2 expression in LNCaP cells after 4 hours treatment with 1nM and 10nM chemerin however this was not statistically significant. After 24 hours chemerin treatment

there was an increase in bcl-2 expression in both cell lines. The increase was more marked in PC3 cells after treatment with 1nM ($p < 0.01$) and 10nM ($p < 0.001$) respectively. There was a 2½ to 3½-fold increase in bcl-2 expression in this cell line after this time period. Less marked but still significantly increased was the 2-3 fold change in bcl-2 expression in LNCaP cells after 24 hours treatment with 1nM ($p < 0.01$) and 10nM chemerin ($p < 0.05$) respectively. The expression of p53 was also reduced in LNCaP cells after 24 hours treatment with 0.1nM chemerin ($p < 0.01$) and 10nM chemerin ($p < 0.05$) respectively. The implications of these results in terms of the effects that have been demonstrated on cell proliferation, invasion, migration and apoptosis will be discussed later.

4.6 Discussion

The aim of these preliminary *in vitro* studies was to explore the role of chemerin and its effect on some of the key steps in cell physiology that promote malignant growth (63) & (64):

1. Self-sufficiency in growth signals
2. Insensitivity to anti-growth signals
3. Evasion of apoptosis
4. Sustained angiogenesis
5. Tissue invasion and metastasis
6. Limitless replicative potential

The experimental data above shows clear evidence that chemerin plays in role in stimulating key steps in carcinogenesis in prostate cancer cells. There is already evidence that chemerin plays a role in angiogenesis causing increased migration and capillary-tube formation in endothelial cells in a time (0-24 h) and dose (0-30nM) dependent manner (47). Angiogenesis is a key step in carcinogenesis as without a sufficient blood supply expanding tumour cells would outstrip their blood supply, essential for cell growth whether normal or deranged. Chemerin appears to clearly influence and increase the proliferation of human prostate

cancer cells (Figure 4-1) after 24 h. In the PC3 cell line there appears to be a dose-dependent significant increase in cell proliferation with the highest rate of cell proliferation being at the highest concentration of chemerin used (10nM). Although in the LNCaP cells the only significant increase in cell proliferation was seen at 1nM chemerin (Figure 4-2) there appeared to be an increase in proliferation at all concentrations of chemerin used in PC3 cells. These results are in keeping with other published data regarding adipokines and prostate cancer cell growth. Higher levels of visfatin (27) and leptin (associated with increasing BMI) and lower levels of adiponectin (associated with decreasing BMI) (19) have been shown to increase PC3 cell proliferation. These results reinforce the hypothesis that adipokines may play a key role in obesity-related prostate cancer. As mentioned in the materials & methods section, the assay used to assess cell proliferation is dependent on the amount of ATP present in the cells. The data suggests that the amount of ATP detected is *directly* proportional to the amount of cells present and is therefore representative of the degree of cellular proliferation. It is possible however, that chemerin may increase the *metabolic* activity in the cells rather than the actual number of cells, leading to increased levels of ATP and an apparent increase in cell proliferation. This is a potential limitation of the assay used and needs to be taken into account when interpreting the results.

Using a cell invasion assay it was not possible to demonstrate any effects on chemerin-induced invasion in either cell line after 24 h (Figure 4-3). There was a slight trend at increased invasion in the LNCaP cell line but this was not significant. As the PC3 cell line is generally more aggressive and grows more quickly it may have been expected that any changes would have been seen in this cell line first but this was not the case. If more time points were used, either shorter or longer, a significant result may have been demonstrated. If 24 h was too short a time point, perhaps cells that would have migrated weren't able to do so in the time allowed. If 24 h was too long a time point cells may have migrated through the reconstituted basement membrane (even those in the basal (0 nM chemerin) group) and so no difference relative to basal would have been demonstrated. The fact that no difference

was demonstrated in cell invasion even in the positive control group (10% FCS) suggests that the optimum time point was not reached. It may have been preferential to use a pre-labelling (kinetic) strategy rather than a post-labelling (end-point) analysis technique. The pre-labelling technique could have permitted multiple time points (4 – 48 h) to be analysed to see whether any difference in cell invasion was present. Due to time constraints and cost issues it was not possible to repeat the invasion assay multiple times.

Figure 4-6 demonstrates the rates of cell migration after 24 h in the human prostate cancer cell lines PC3 and LNCaP. Only in the PC3 cell line was a significant increase in cell migration demonstrated ($p < 0.001$) at the two doses of chemerin used: 1 and 10nM. Similar to the cell proliferation assay the rate of cell migration appeared to increase in a dose-dependent manner, with the highest rate of migration being found in the highest dose of chemerin (10nM). Had the migration assay been carried out for a longer time period (36-48 h) in the LNCaP cell line a positive correlation may have been demonstrated. The fact that no positive correlation was demonstrated after 24 h in the LNCaP cell line may well reflect the fact that these cells are generally slower growing than PC3 cells. As with the cell invasion assay no positive effect was demonstrated with the positive control (10% FCS) suggesting that the optimum time point for migration in this cell line was not reached. It could be argued that the invasion ("wound scratch") assay is also a surrogate marker of cell proliferation. Fundamental to the principle of the assay is the assumption that to migrate in to the "wound" individual cells must detach and then re-attach in the wound (i.e. where there are no cells). Having seen that there is increased cell proliferation in PC3 and LNCaP cells it could be proposed that simply the increased rate of proliferation of cells and therefore the expanding mass of cells simply expands in to the wound mimicking migration. If this were to be true then one might have expected an increase in cell migration in *both* cell lines after 24 hours however there was an increase in migration of PC3 cells only. LNCaP cells do not grow in a uniform monolayer but grow in clusters or clumps unlike PC3 cells which tend to grow in a more uniform monolayer. It may not be possible to reciprocate the increase in cell

proliferation in the migration assay as LNCaP cells may not grow along the base of the well into the wound but upwards therefore causing an increase in cell proliferation *without* an apparent increase in cell migration.

Prevention of apoptosis is a key step in the propagation of carcinogenesis and induction of apoptosis is a key mechanism of anti-cancer drug therapy. Apoptosis appears to play a pivotal role in cancer development and treatment. In the PC3 cell line there appeared to be a reduction in apoptosis after 24 h at the lower concentration of chemerin (0.1nM); however this result was not statistically significant (Figure 4-9). There appeared to be satisfactory evidence that the assay was set up appropriately as the positive control (H_2O_2) showed an expected increase in apoptosis after 4 h as was the case in the LNCaP cell line after the same time period. It would have been expected that if there were to be any changes in apoptosis in the PC3 cell line it would have been demonstrated at the time points used. A statistically significant *reduction* in apoptosis was clearly demonstrated in the LNCaP cell line after 4 and 12 h at higher concentrations of chemerin (1 and 10nM) (Figure 4-12). The apparent reduction in apoptosis at 24 h was not statistically significant but is in keeping with the rest of the data. At no time point or dose of chemerin was there an apparent *increase* in the rate of apoptosis in either cell line (apart from in the LNCaP cell line after 12 h treatment with 0.1nM chemerin).

In order to assess which intra-cellular mechanism induced these changes, (increased proliferation, migration and reduced apoptosis) the levels of four key regulatory proteins (p53, Bax, bcl-2 and bcl-2 L1) in cellular processes were evaluated in both PCa cell lines (PC3 and LNCaP) at two time points (4 and 24 hours). The most pertinent and significant changes appeared to be confined to the levels of p53 and bcl-2 expression. After 24 hours treatment with chemerin in both cell lines there was a significant *increase* in expression of bcl-2 with increasing levels of chemerin concentrations suggesting that the changes previously mentioned were mediated through bcl-2 stimulation. Bcl-2 is one protein of a family of proteins that are involved with multiple cellular processes including proliferation,

growth and apoptosis. The finding that bcl-2 expression is increased at the mRNA level in both cell lines (androgen dependent and independent) is an interesting one and suggests that chemerin may play a role in both disease states. It may lead to increased growth of PCa cells in the early stages (androgen dependent) of PCa and also play a role in the progression of the disease to the androgen independent state. I must be noted that even though there was a significant increase in cell proliferation in both cell lines after 24 hours there was no such statistically significant change in apoptosis after 24 hours. There appeared to be a reduction in apoptosis in the LNCaP cell line after 24 hours however this did not reach significance. Generally bcl-2 is referred to as an anti-apoptotic protein, (65) however it does also appear to exert some effects on cellular proliferation and may therefore be responsible for the increase cellular proliferation seen in both cell lines after 24 hours. It may be that the changes demonstrated in proliferation are simply through the ability of chemerin acting through bcl-2 to prevent apoptosis and therefore there is an apparent increase in cellular proliferation.

Dysfunction or deregulation of bcl-2 has been implicated in numerous malignancies including breast (66), melanoma (67) and prostate (68). Generally it appears that bcl-2 prevents apoptosis and therefore if it were to be up-regulated for whatever reason then one might expect to see a reduction in apoptosis and increased longevity of a cell. Bauer et al., demonstrated by immunohistochemistry (IHC) that in 175 RRP specimens that expression of bcl-2 was significantly associated with disease recurrence following radical surgery. Although the expression of bcl-2 was not particularly high (n = 47, 26.9%); in clinically localised PCa its presence was an independent prognostic factor as demonstrated by Kaplan-Meier survival analysis and was associated with a significantly worse recurrence rate at five years (67.0% vs 30.7%). Conversely those samples that stained negatively for the tumour suppressor gene (TSG), p53 lead to lower biochemical recurrence rate of 22% at 5 years. Another study examining bcl-2 and p53 expression in 137 radical prostatectomy specimens demonstrated that overexpression of bcl-2 was significantly higher in T3 tumours compared

with T2 tumours (31% vs 5%) (68). This suggests that bcl-2 expression is associated with a poorer outcome as T3 disease is more likely to lead to biochemical recurrence and a reduction in long-term survival. It must also be noted that a higher Gleason score was also associated with pT3 disease and lymph node positive disease suggesting that more advanced disease is multi-factorial however bcl-2 positivity itself was not associated with either T stage or lymph node status. As previously discussed negative staining for bcl-2 was associated with a better prognosis in terms of disease-free survival. It is unclear from this study what exactly is the reasoning behind the reduced survival following surgery however it is thought to be unlikely due to bcl-2-induced hormone resistance as very few men in this study underwent ADT. Bcl-2 expression appeared to have little impact on survival for men who subsequently underwent salvage radiotherapy suggesting that radiotherapy-induced apoptosis is also unaffected. These authors also report that samples staining positively for p53 showed a reduced survival although this did not reach significance with much fewer samples staining positive for p53 than negative. Knowledge of bcl-2 activation in advanced PCa has led to the potential use of the bcl-2 antagonist ABT-737, in advanced PCa (69). Interestingly when ABT-737 is used as a single agent, limited apoptosis is induced *in vitro* however when agents targeting Mcl-1 are used in combination with ABT-737, such as etoposide, paclitaxel and cisplatin, apoptosis is promoted in a novel tumour explant system. Mcl-1 is an anti-apoptotic protein and another member of the bcl-2 family and it is suggested that it may be responsible for progression of PCa in advanced stages.

The process of apoptosis is dependent on the functioning of p53, a TSG that regulates the active process of programmed cell death (70). The finding that expression of p53 is reduced in LNCaP cells after 24 hours at 0.1 and 10 nM of chemerin is therefore potentially a significant finding. It must be noted however, that no significant reduction in apoptosis was demonstrated in LNCaP cells after 24 hours. Since PC3 cells lack active or functioning p53 (71) it may come as no surprise that no changes in apoptosis were demonstrated in this cell line and certainly there was no change in expression of p53 either after 4 or 24 hours

treatment with chemerin stimulation. These authors also demonstrate that mutations of p53 are present in multiple PCa cell lines and p53 is widely considered to be a key TSG in many cancers, including PCa. It has subsequently been demonstrated that apoptosis in PC3 cells is likely to be independent of p53. Bataller et al., demonstrated that oxidative stress–induced apoptosis in PC3 cells is mediated by p21 (72). H_2O_2 appears to induce apoptosis in this cell line by p21 acting as a substrate for caspase-3 (a member of the caspase family of cysteine proteases that are key facilitators of apoptosis). The lack of p53 in this cell line means that PC3 cells (a model of more aggressive PCa) are less likely to be induced to enter apoptosis through the stimulus of oxidative stress and are more likely to persist. This may partially explain why, when PCa enters the androgen-independent stage of the disease it is much more difficult to control and why it is invariably lethal.

5 The effect of chemerin on signal-transduction pathways and matrix metalloproteinases (MMP) in Prostate Cancer

5.1 The role of the phosphatidylinositol 3-kinase (PI3K)/AKT (Protein Kinase B) pathway in prostate cancer

The PI3K/AKT pathway is a key pathway in a number of cellular processes including cell proliferation, apoptosis and glucose metabolism. AKT is also a negative regulator (inhibitor) of apoptosis and migration and is sometimes referred to as Protein Kinase B (PKB). Activation of this pathway is associated with numerous malignancies (73) including PCa. Increased AKT activity is associated with PCa progression in an androgen-independent cell line LNAI (74). LNAI cells were developed from xenograft tumours of the normally androgen *sensitive* cell line LNCaP and so were positive for the androgen receptor and PSA. The reason for the development of this cell line was that these cells readily form tumours regardless of the androgen status of the surroundings and therefore more closely mimic *in vivo* scenarios. In the LNAI cell line, particularly the second generation LNAI cell lines (more aggressive LNAI cells such as T1.8, T1.16, and T2.11), total AKT expression was unchanged compared to LNCaP cells. Interestingly AKT activation (particularly Ser-473 phosphorylation) was significantly higher in the second generation LNAI cell lines as demonstrated by WBA. Increased AKT activity in PCa could be related to the fact that PTEN (Phosphatase and tensin homolog), a negative regulator of the AKT pathway, is frequently inactivated in PCa (75). Lower expression of PTEN, as demonstrated by IHC, appears to be associated with a higher Gleason score (≥ 7) and a more advanced stage of tumour (≥T3b). A study by Malik et al., evaluated the strength of phospho-AKT staining in higher Gleason scores (8-10) in paraffin-embedded PCa samples and compared this to samples containing lower Gleason score (2-7) tumours and prostatic intraepithelial neoplasia (PIN) lesions (76). Strength of staining was characterised according to the following grades: weak (0 to +1) and strong (+2 to +3). Out of the 25 cases of high Gleason score 23 stained

strongly for phospho-AKT (92%) whereas this was only the case in 10 out of 49 (20%) lower Gleason score tumours. In the PIN lesions all 51 samples were categorised as staining weakly. The difference between the high Gleason score tumours, the lower Gleason score tumours and the PIN lesions was highly significant. There is hope that targeting either the pathway itself or molecules within the pathway may lead to a potential therapeutic target for HRPC. The PI3K inhibitor: LY294002 inhibits cell proliferation in PC3 (48-72h) and DU145 (24-72h) prostate cancer cells at both 10 and 20μM compared with serum alone. G_1 cell cycle arrest is also induced in both PC3 and DU145 prostate cancer cells at time points up to and including 48h with both concentrations of the same PI3K inhibitor (77). AKT staining intensity, as determined by IHC is significantly higher in malignant prostate tissue than in benign prostatic tissue (78). In 56 prostate samples removed for PCa 176 tissue areas were isolated comprising benign prostatic tissue (BPT), PIN and cancer specimens. The tumour patterns demonstrated ranged from Gleason pattern 2 to 5. AKT expression was demonstrated in all prostate tissue categories and appeared to be localised to the cytoplasm. Staining intensity was classified as being weak, moderate or strong. In the BPT samples the majority stained weakly for AKT (34/54: 63%) with only 2 out of 54 samples staining strongly for AKT. In the PCa samples staining intensity increased, for example in the Gleason pattern 4 samples 5/11 (46%) stained strongly and this increased to 5/6 (83%) for Gleason pattern 5 disease. No weak staining was demonstrated for AKT in Gleason pattern 4 or 5 disease. There was no correlation between AKT staining and the pathological tumour stage (pT) which is unfortunate as the ability of a tumour marker to accurately predict the pathological or post-operative tumour stage from clinical or pre-operative data would be invaluable in the management of PCa. The ability of a tumour marker to accurately predict those at risk of more advanced disease in PCa is a key area of research. There was also a significantly higher staining intensity of AKT in men with a higher pre-operative PSA than in those with a lower PSA (≥10 vs <10ng/ml), but there was no correlation with Gleason score on an individual patient basis. Men with a pre-operative PSA of greater than or equal to 10 tend to be assigned to a higher risk classification group in any case and these men are

unlikely to be offered an option of surveillance for their PCa (79). The finding that there is increased AKT expression pre-operatively is unlikely to alter the management in a patient with PCa on an individual patient basis. Molecules in the PI3K/AKT pathway could therefore be potential prostate cancer markers for patients at risk of developing biochemical recurrence following radical treatment and those with HRPC. Therapeutic manipulation of thePI3K/AKT pathway is the subject of vigorous on-going clinical trials with the potential for significant survival benefits in men with PCa, mainly those with HRPC who have exhausted the proven Taxotere-based chemotherapy agents such as Docetaxel. Time will tell whether the pathway and its relevant proteins are going to be useful for future prognostic markers or a viable therapeutic target in PCa. To this authors knowledge no published data so far shows chemerin causing either activation or deactivation of the AKT pathway in PCa. The adipokine, adiponectin has been show to activate mammalian target of rapamycin (mTOR) via the PI3K/AKT pathway in PTEN-deficient LNCaP cells (80).The AMP-activated protein kinase (AMPK) pathway is also activated by adiponectin, however the dominant effect on the mTOR pathway was via the PI3K/AKT pathway. Chemerin has been shown to activate the AKT pathway in a dose dependent and time dependent manner in human endothelial (47), and in human articular chondrocyte cells (31). Chemerin has been shown to regulate energy metabolism via the AKT pathway in a *negative* way in differentiated C2C12 (mouse myoblast) cells at 30 and 60 minutes. The published data thus far suggests, therefore a complex, and at times contradictory role for chemerin-mediated AKT activation in cell metabolism. The role of chemerin and its effects on the PI3K/AKT pathway in prostate carcinogenesis therefore remains to be elucidated.

5.2 The role of the Mammalian target of rapamycin (mTOR) pathway in prostate cancer

The mammalian target of rapamycin (mTOR) pathway has been shown be activated in PCa and is a potential future therapeutic agent in HRPC as well as another potential prognostic marker. The mTOR inhibitor: Temsirolimus in a multicentre, phase III trial has been shown

to increase overall and progression-free survival in patients with metastatic renal cell carcinoma compared to Interferon-α (IFN-α) alone, or in combination with IFN-α (81). The drug appears to be better tolerated with fewer adverse reactions reported compared to IFN-α. The drug was approved by both the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for use in metastatic renal cell carcinoma in 2007. As yet the use of mTOR inhibitors in any stage of PCa has not reached the same stage as for other cancers and currently the outcome of several phase I/II clinical trials are awaited (82). Expression of the mTOR protein and multiple proteins within the mTOR pathway including 4E-binding protein 1 **(**4EBP1), phosphorylated 4EBP1 (p-EBP1), S6 and phosphorylated S6 (p-S6) were shown to be significantly higher, especially 4EBP1, in PIN sections compared with normal prostate tissue in the majority of samples as demonstrated by IHC (83). Expression of PTEN, a negative regulator of the mTOR pathway, was noted to be predominantly expressed at lower rates in cancerous compared to benign tissue. How this increased expression of 4EBP1 affects long-term outcomes in terms of overall and disease-free survival in men with PCa remains to be elucidated. The mTOR pathway may play a key role in the survival of prostate cancer cells in the low androgen environment in men undergoing ADT. A study showed that sub-baseline mTOR levels increased AR protein levels but only in a **low** testosterone environment (0.03nM) (84). This may in part explain the ability of prostate cancer cells to survive and ultimately proliferate in the low testosterone levels found in men undergoing ADT whose PCa ultimately escapes hormonal control. One of the most comprehensive studies looking at the role of the mTOR pathway in PCa evaluated the role of 67 genes from the mTOR pathway nested within the European Prospective Investigation on Cancer (EPIC) study, a study with 150 000 men from 10 Western European countries (85). Two single-nucleotide polymorphisms (SNP) were detected in the PRKCI gene that encodes for Protein kinase C iota type – which appears to confer a lower risk of PCa. There appears to be no SNP that affects other genes in the mTOR pathway that have been mentioned earlier.

5.3 The role of extracellular signal-regulated kinase (ERK) signalling in prostate cancer

Mitogen-activated protein kinases (**MAPK**) are key mediators in regulating a multitude of cellular processes including proliferation and apoptosis. The three commonest MAPK are as follows (86) & (87):

- Extracellular signal-related kinases (**ERK**)
- c-Jun N-terminal kinases/stress-activated protein kinases (**JNK/SAPKs**)
- **p38** MAPK

Activation of each of these mediators represents the final step in their respective pathways which will then trigger the corresponding downstream cellular effects. In PCa ERK signalling is thought mainly to play a role in cellular growth, differentiation and development. Indeed, in LNCaP cells, Epidermal Growth Factor (EGF)-dependent cellular proliferation appears to be dependent on the ERK pathway (88). Stimulation of LNCaP cells with EGF lead to the phosphorylation of ERK whereas DHT did not. This phosphorylation of ERK was noted after as little as five minutes and began to fall to near basal levels after two hours. These results suggest that cell proliferation in PCa is mediated by multiple and heterogeneous cellular pathways. In PC3 cells, EGF and lysophosphatidic acid (LPA) stimulation both lead to an increase in cellular proliferation with corresponding phosphorylation of the ERK pathway. As with ERK phosphorylation in LNCaP cells, EGF and LPA activation of ERK in PC3 cells was noted after 5 minutes and persisted up to 2 hours. ERK activation appeared to be sustained with LPA treatment compared to EGF. The MEK (an immediate upstream kinase responsible for the activation of ERK) inhibitor: PD98509 lead to a significant reduction in EGF-induced cellular proliferation in LNCaP cells. A similar reduction in EGF and LPA-related cellular proliferation was noted in PC3 cells after treatment with the tyrosine kinase inhibitor AG1478. In contradiction to this evidence it has been shown that treatment of androgen dependent LNCaP cells with varying

doses of DHT (0.01 – 10nM) does in fact lead to *activation* of the ERK1/2 pathway (89). It may be that prolonged activation of this pathway leads to an evasion of apoptosis that is indicative of HRPC and advanced disease. Interestingly DHT levels tend to be lower in the stages of advanced disease due to use of ADT therefore ERK activation and subsequent evasion of apoptosis may not be mediated through the DHT and the AR. The use of Casodex® (AstraZeneca, London UK), an anti-androgen drug commonly used in PCa at lower concentrations (1nM) in combination with DHT worked as an antagonist contributing to ERK 1/2 phosphorylation. This may in part be a reason why advanced PCa can progress despite apparently completely blocking the stimulation of androgens.

Iacopino et al., evaluated the role of EGF and Leuprorelin acetate (LA – a Gonadotrophin releasing agonist commonly used for ADT in PCa) on cellular proliferation and ERK activation (90). At higher concentrations of EGF (≥5 ng/ml) there was a significant increase in cellular proliferation of both LNCaP and PC3 cells. When 10ng/ml EGF was used (the concentration that appeared to give the greatest increase in cellular proliferation in both cell lines) together with varying concentrations of LA there appeared to be a reduction in EGF-dependent cellular proliferation in both cell lines. These authors demonstrated by using WBA that in only PC3 cells was pERK1/2 as well as ERK1/2 expressed at *basal* levels. This basal level of pERK1/2 expression was not present in LNCaP cells. Conversely using immunocytochemical analysis it was demonstrated that pERK1/2 was present in both cell lines although it was not possible to differentiate between ERK1 and ERK2 (p42 MAPK and p44 MAPK). There appeared to be a heterogeneous staining pattern of pERK1/2 with nuclear and cytoplasmic staining present. EGF treatment leads to activation of ERK1/2 in a time dependent manner after 5 minutes treatment as demonstrated by WBA in LNCaP cells. The maximum effect of ERK1/2 phosphorylation was noted after 60 minutes and levels fell back over the following 4 hours. This data suggests that it may well be that stimulation of the ERK1/2 pathway is an important pathway in cellular proliferation, in LNCaP cells at least. Inhibition of phosphorylation of ERK1/2 by EGF receptor activation was

demonstrated using the EGF-inhibitor: AG1478. After 5 minutes a 90% reduction of EGF-dependent ERK1/2 phosphorylation was demonstrated which gradually diminished after 60 minutes and was lost after 240 minutes (4 hours).Treatment of PCa cells with EGF and LA generally lead to a reduction of ERK1/2 activation at most concentrations of LA. Further analysis seemed to suggest that lower concentrations of LA (10^{-11}M) lead to a greater reduction in ERK1/2 phosphorylation. Conversely in the androgen-insensitive cell line: PC3 LA treatment tended to lead to activation of ERK1/2. The addition of EGF to LA-treated PC3 cells lead to a more rapid and persistent increase in ERK1/2 activation. This may explain how LA is able to control PCa growth, albeit temporarily, by reducing cellular proliferation through inhibition of the ERK1/2 pathway. It may also explain in some part the inability of LA (or similar leutenizing-hormone releasing-hormone (LHRH)) – agonists to control PCa in men who have progressed to the castrate-resistant stage of the disease where cellular proliferation is "out of control". Kinkade et al., again highlight a role for ERK signalling in PCa; and in particular the development of HRPC in conjunction with mTOR-signalling (91). Using the Nkx3.1; PTEN pre-clinical mutant mouse model these authors evaluate the role of simultaneous mTOR and ERK inhibition in PCa progression *in vivo*. The reason that this mouse model was used was that it follows a highly reproducible model of the disease from PIN initially to hormone sensitive and later to hormone refractory PCa under androgen ablation conditions (92). This model also demonstrates evidence of ERK MAPK, as well as AKT/mTOR activation making it an ideal model for examining the authors' hypothesis.

The pathways above were therefore examined in PCa cells in order to determine if chemerin modulates their activation and are possible mediators of obesity-related prostate carcinogenesis.

5.4 The effects of chemerin on matrix metalloproteinases (MMP) in prostate cancer.

Matrix metalloproteinases (MMP) are potential key enzymes in disrupting the cell-to-cell adherence mechanisms (degradation of the basement membrane and extracellular matrix (ECM)) in organs and their activity is potentially a key step in the development of metastatic disease in many cancers including PCa. Their activity has been demonstrated in numerous other malignancies and has been the subject of previous research and investigation (93). It is thought that the development of MMP inhibitors may be a key therapy in either the prevention of, or the treatment of metastatic PCa. Marimastat (BB-2516), a MMP inhibitor (second generation) and tetracycline's are some of the drugs that have shown some promise in the treatment of PCa in pre-clinical trials. Over 20 MMP have thus far been demonstrated and many are up regulated in specific cancer types. In normal conditions generally expression of MMP are at low levels but sometimes an increased level of expression is seen in certain circumstances such as embryogenesis and in the repair of wounds and bone. Stearns et al., demonstrate by ELISA that levels of MMP-2 and MMP-9 are directly correlated with the ability of PCa cell lines to induce angiogenesis through microvessel formation (94). This experimental data suggests that MMP not only play a key role in metastasis through the effects on the basement membrane and ECM but also through the induction of angiogenesis, as discussed earlier in chapter 4.6 being one of the "hallmarks of cancer". These authors also demonstrate the effects of interleukin (IL)-10 and its effects on microvessel activity through MMP manipulation. It was demonstrated that IL-10 15 ng/ml lead to a significant increase in tissue inhibitor of metalloproteinase (TIMP)-1 secretion which in turn lead to a decrease in MMP-2 and MMP-9 levels. This therefore suggests a mechanism by which MMP activity can be inhibited through IL-10 stimulation. It appears that two common forms of MMP implicated in PCa are MMP-2 and MMP-9 which appear to be commonly associated with metastasis to bone, lymph nodes and lung (95). Four sub-types of protease-activated receptors (PAR 1-4) have been identified and it is thought that

activation of these GPCR leads to increased activity of MMP. Indeed stimulation of PAR-1 and PAR-2 leads to the increased activity of MMP-2 and MMP-9 in the PCa cell lines PC3, DU-145 and LNCaP as demonstrated by MMP activity assays (96). It is interesting to note that experimental, *in vitro* data does not necessarily translate into clinical *in vivo* data. In 278 RRP specimens, a study evaluating the prognostic ability of several MMP (-2, -3, -7, -9, -13, -19) to accurately predict overall and disease-specific survival as well as disease recurrence; demonstrated that only MMP-9 was found to be an accurate prognostic marker. In contradiction to earlier evidence, an *increased* expression of MMP-9 was associated with better overall and disease-specific survival as well as delayed recurrence as demonstrated by a tissue microarray (TMA) (97). No significant prognostic information was gleamed from evaluating the other MMP in terms of survival or recurrence. It may be that there is actually no correlation between expression of MMP and survival in PCa or simply that this study failed to demonstrate a significant correlation.

5.5 Results

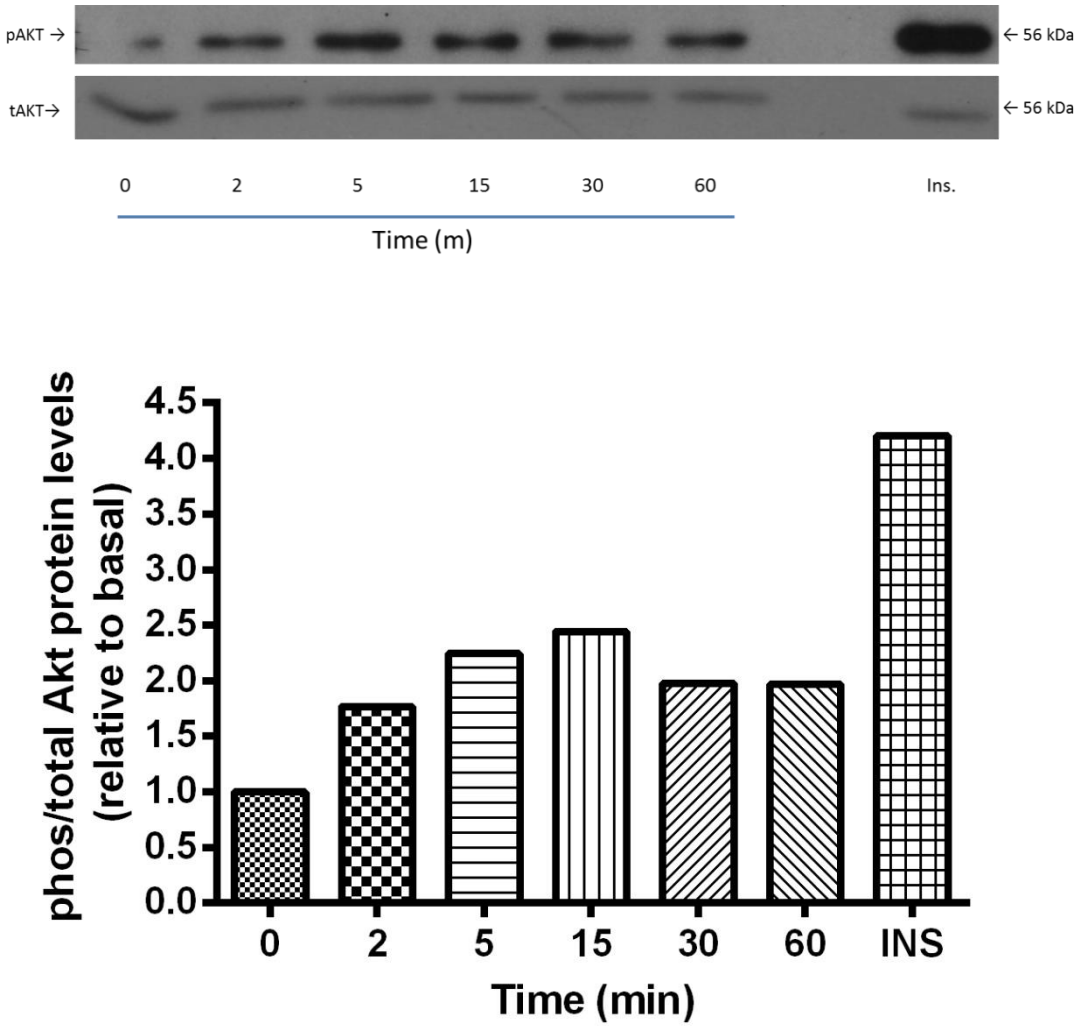
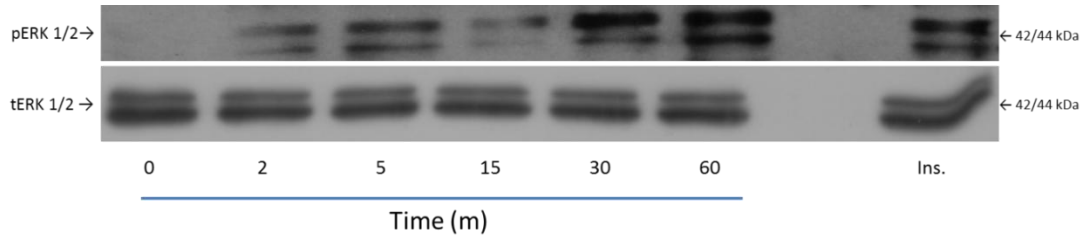


Figure 5-1: Representative western blot (above) and graph (below) demonstrating the effects of chemerin on AKT activation in PC3 cells after differing time points, (n = 1).



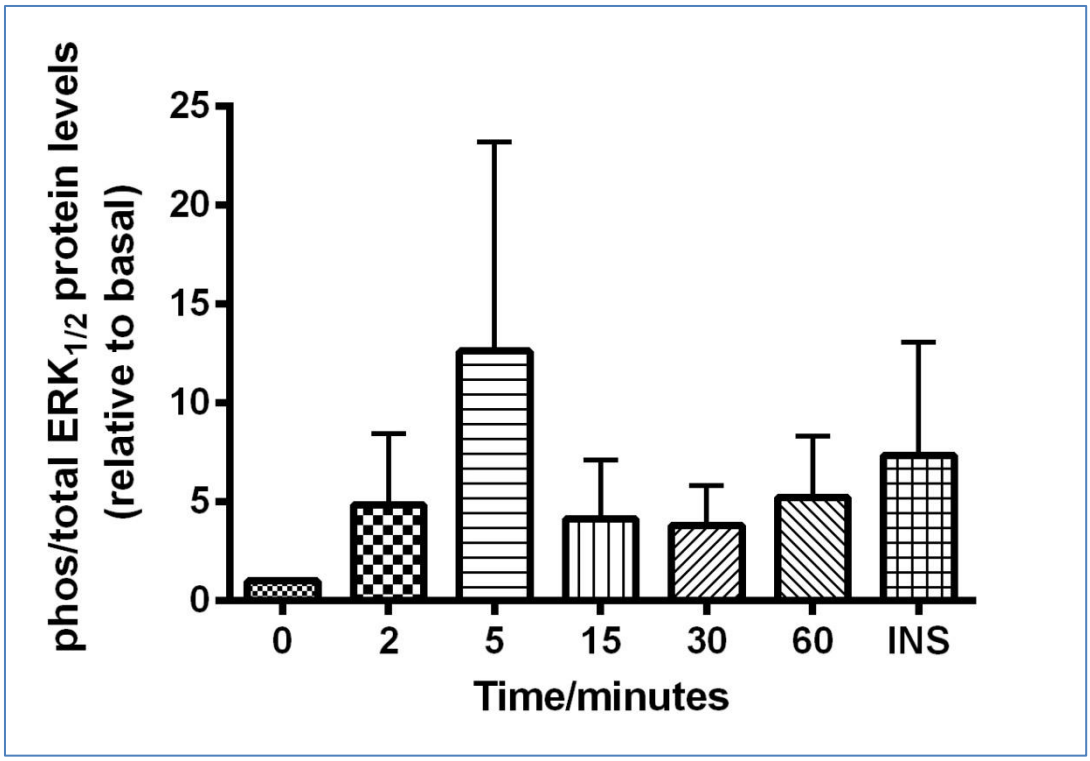


Figure 5-2: Representative western blot (above) and graph (below) demonstrating the effects of chemerin on ERK 1/2activation in PC3 cells after differing time points, (n =2).

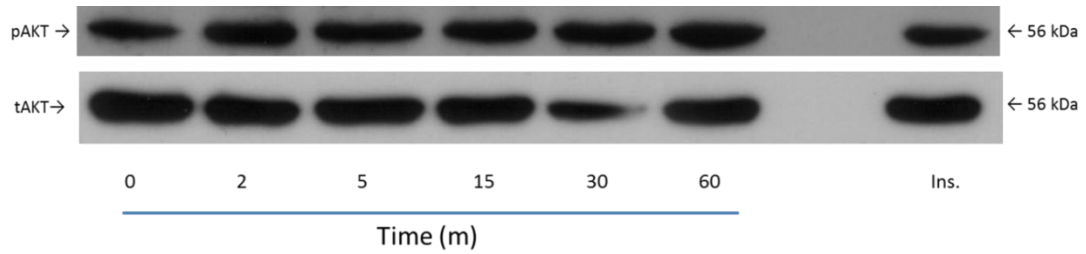


Figure 5-3: Representative western blot demonstrating the effects of chemerin on AKT activation in LNCaP cells after differing time points.

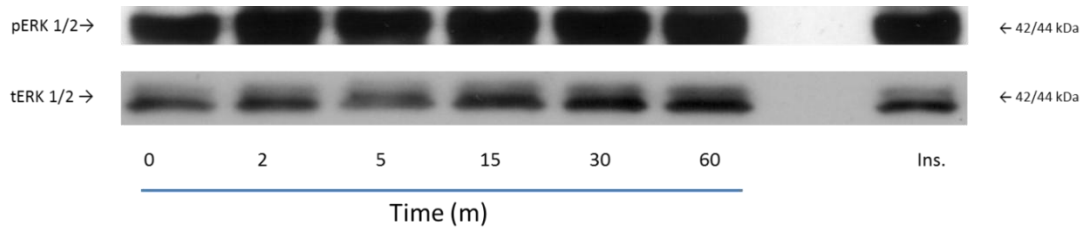


Figure 5-4: Representative western blot demonstrating the effects of chemerin on ERK 1/2 activation in LNCaP cells after differing time points.

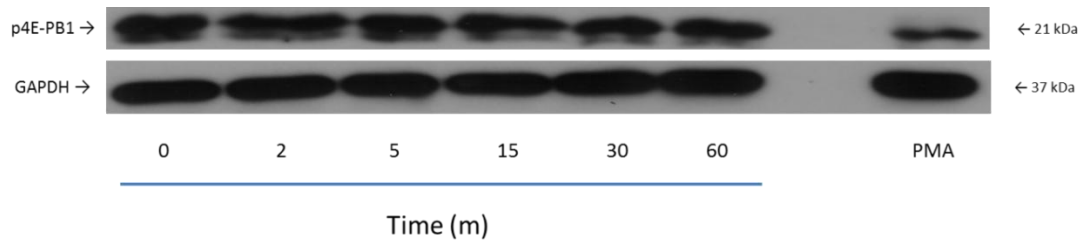


Figure 5-5: Representative western blot demonstrating the effects of chemerin on 4E-PB1 activation in PC3 cells after differing time points.

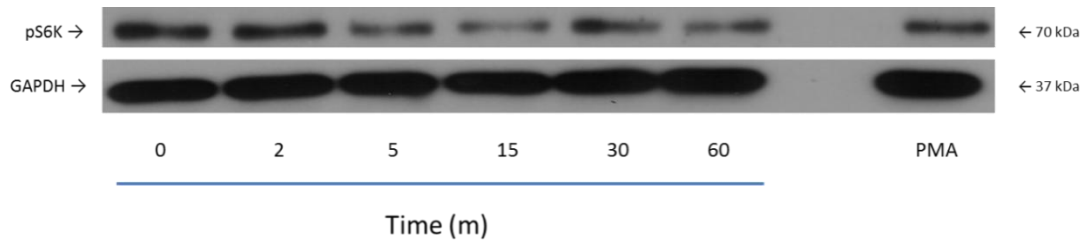


Figure 5-6: Representative western blot demonstrating the effects of chemerin on S6K activation in PC3 cells after differing time points.

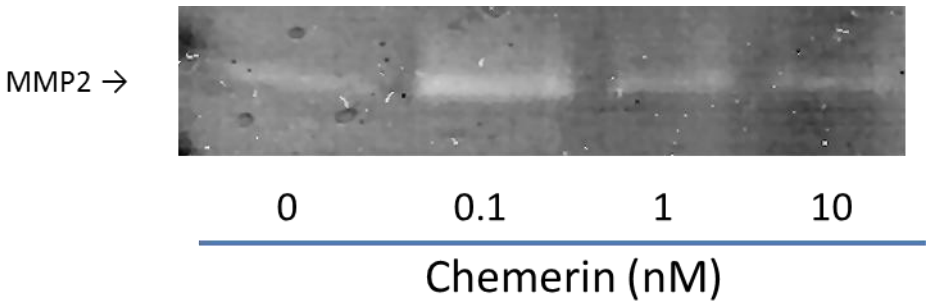


Figure 5-7: Gelatin zymograph demonstrating MMP2 activity in supernatants of PC3 cells after 24 hours treatment with chemerin.

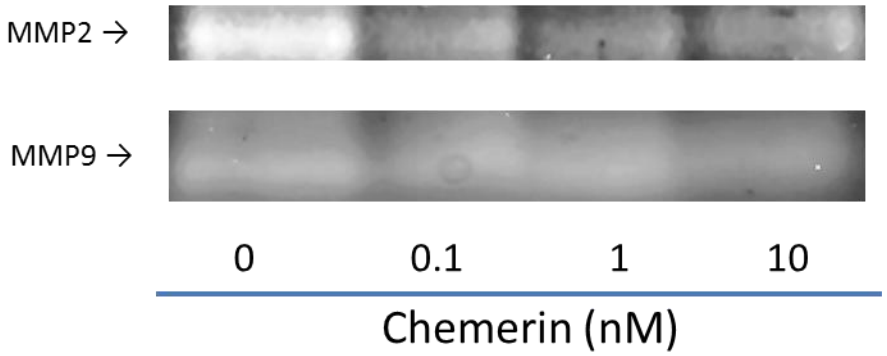


Figure 5-8: Gelatin zymograph demonstrating MMP2/9 activity in supernatants of LNCaP cells after 24 hours treatment with chemerin.

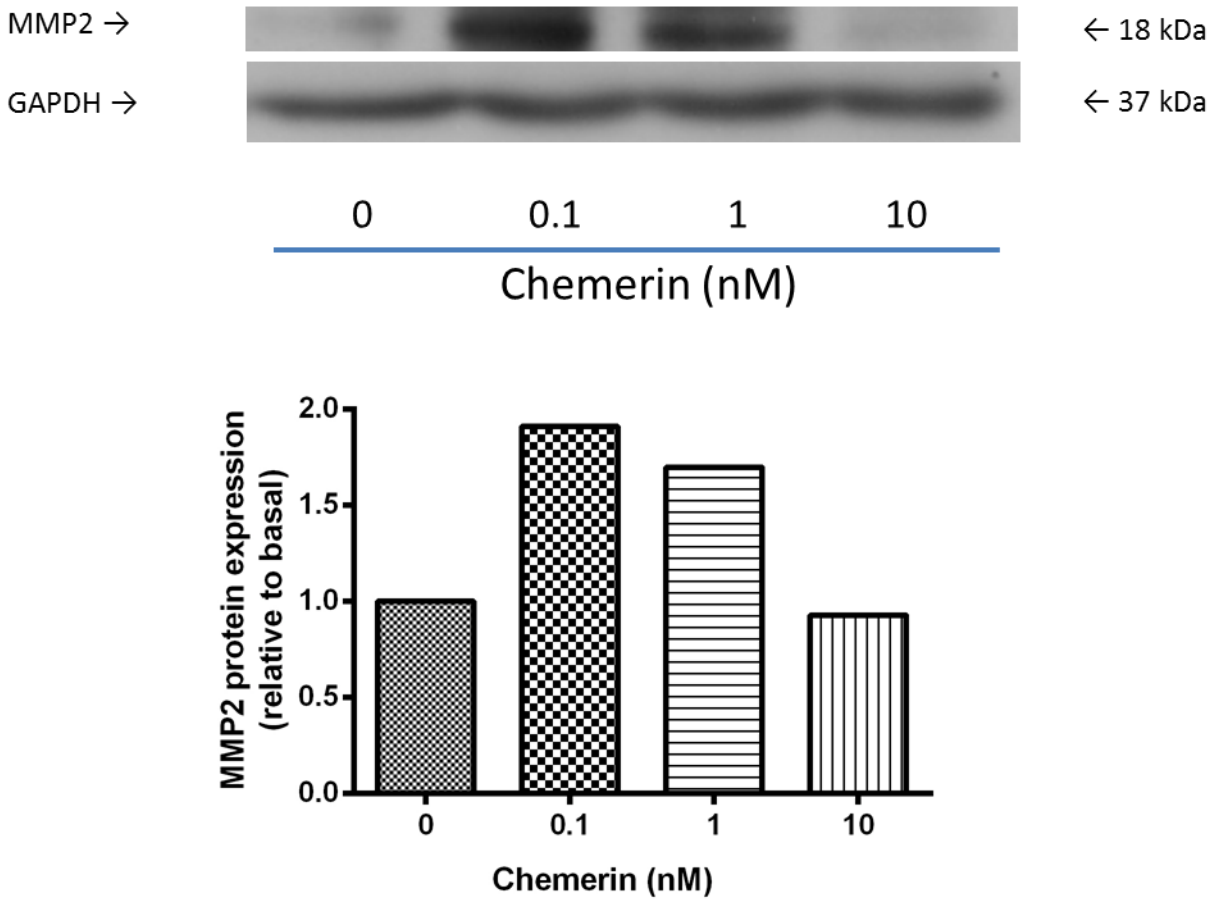


Figure 5-9: Representative western blot (above) and graph (below) demonstrating the effects of chemerin on MMP2 protein expression in LNCaP cell lysates (n = 1).

In the PC3 cell line there appeared to be activation of both AKT and ERK1/2 as demonstrated by WBA, however this failed to reach statistical significance. In both AKT and ERK 1/2 there was activation of each pathway with chemerin stimulation after as little as 5 minutes and this activation was sustained for each protein. After stimulation of chemerin for 15 minutes activation of ERK 1/2 appeared to drop back down to near basal levels before rising again after 30 to 60 minutes. It is unclear what may have led to this finding and the possible reasons will be discussed later. mTOR pathway activation was examined by looking at the phosphorylation of two key downstream targets of mTOR: 4EBP1 and S6K. There appeared to be no major activation or inactivation of either protein within the 60 minute treatment time that was used during these experiments in PC3 cells. This is the reason why no graphic representations of these proteins are demonstrated underneath the western blots. With regards to ERK 1/2 and AKT activation in the LNCaP

cell line there appeared to be no evidence of any activation of either protein at the time points evaluated (1-60 minutes). Unfortunately due to time constraints and the difficulties mentioned with LNCaP cell culture it was not possible to evaluate mTOR pathway activation through 4E-PB1 and S6K phosphorylation in this cell line.

5.6 Discussion

As mentioned in the introduction ERK and AKT are key cellular proteins and their activation is a key step in proliferation in PCa cells. They appear to play a key role in prostate carcinogenesis and that is why activity of these proteins was analysed in the PCa cell lines: PC3 and LNCaP. Only in the PC3 cell line did there appear to be any AKT activation after as little as 2 minute's treatment with 1nM chemerin. This response was sustained and persisted until at least 60 minutes (the end of the experiment). There was no such similar response in the LNCaP cell line. It may be possible that a response may have been demonstrated in the LNCaP cell line at a different concentration of chemerin, possibly less than 1nM chemerin. As demonstrated in the western blots above (Figure 5-3) there was activation of AKT instantaneously at 1nM chemerin and so a lower concentration of chemerin may have allowed a demonstration of AKT activation during the time points used during the experiment. Similar to AKT activation there was evidence of ERK1/2 activation in the PC3 cell line but not in the LNCaP cell line. The response was noticed after 5 minutes and persisted until the end of the experiment (60 minutes, apart from a dip to near basal levels after 15 minutes). No response was demonstrated in the LNCaP cell line with regards to ERK1/2 activation. As already discussed, this may be a case of not demonstrating ERK1/2 activation through use of the "correct" concentration of chemerin or time-point.

In the PC3 cell line activation of key proteins in the mTOR pathway, 4E-PB1 and S6K, were evaluated using WBA. As demonstrated with the representative western blots above no activation of the proteins in this pathway was demonstrated. As previously mentioned had a lower concentration of chemerin (<0.1nM) been used some level of activation may have been demonstrated. Due to time constraints it was only possible to evaluate activation of the

mTOR pathway at this specific concentration. No effects were demonstrated in the LNCaP cell line with regards to mTOR pathway activation either (data not shown).

As previously mentioned it has been demonstrated that MMP 2 and 9 are expressed in the PCa cell lines PC3 and LNCaP. This is why I was interested to evaluate the effect that differing concentrations of chemerin had on MMP activity in PCa cells and to see whether chemerin may play a role in obesity-related PCa. In the PC3 cell line zymography demonstrated only MMP 2 activity in the supernatants after 24 hours treatment with chemerin. The reason for this is unclear but may be due to technical difficulties with the gelatin zymography assay. It is unlikely that MMP 9 is absent in the PC3 cell line as it has been demonstrated previously. With regards to MMP 2 expression there appeared to be an increase in expression after 24 hours with 0.1nM chemerin treatment, whereas expression of MMP 2 with 1/10nM chemerin appeared to be near basal (0nM). In the LNCaP cell line MMP 2 & MMP 9 activity was demonstrated. Interestingly with regards to MMP 2 activity this appeared to be highest at basal (0nM) levels of chemerin. MMP 9 activity appeared unchanged when stimulated with chemerin in LNCaP cells. One may have expected that if MMP were to play a key role in the development of metastatic disease, more likely with aggressive, hormone-refractory PCa, then it may have been expected that the effects would have been demonstrated in the less aggressive, hormone-sensitive cell line: LNCaP rather than the hormone-insensitive cell line PC3. It may be that the changes demonstrated in the PC3 cell line were simply down to chance and actually no effects were demonstrated with regards to MMP 2/9 expression after treatment with chemerin in either cell line. MMP 2/9 activity as demonstrated by WBA in PC3 and LNCaP cell lysates was less successful. Only MMP 2 activity in the lysate of LNCaP cells was demonstrated. No MMP 2/9 activity in the lysates of PC3 cells or MMP 9 activity in LNCaP cells was demonstrated by WBA. MMP 2 activity in the LNCaP cell line appeared to increase with 0.1/1nM chemerin in particular. Levels with 10nM chemerin stimulation appeared to be near basal levels. The pattern appeared to be fairly similar to the pattern of expression of MMP 2 in the supernatants of the

PC3 cells demonstrated by zymography. Of course these findings may be simply down to chance but there does appear to be a trend of increased MMP 2 activity and expression in PCa cell lines with chemerin with respect to basal.

The data presented above appears to suggest that the adipokine chemerin modulates certain key proteins and pathways in cellular proliferation, growth and metastasis. These mechanisms are well described in the literature with regards to prostate carcinogenesis and are key steps in the development of PCa. The data presented, although not overwhelming is interesting and does suggest that further work is needed in this area to understand the mechanisms behind obesity-related PCa.

6 The effect that chemerin has on anterior gradient 2 (AGR2) expression

6.1 AGR2 and prostate cancer

The anterior gradient 2 (AGR2) gene, the human analogue of the secreted Xenopus laevis proteins XAG-1/2 (also known as hAG-2 or Gob-4) is implicated in the pathogenesis of numerous adenocarcinomas. AGR2 protein is expressed in the cement gland (an ectodermal organ in the head) and plays an important role in early development and antero-posterior fate determination. It is located on chromosome/loci 7p21. Fletcher et al., demonstrated its potential role in hormone positive breast cancer previously (98). Using IHC on human breast cancer specimens AGR2 was found in 48/58 samples (83%). Staining appeared confined to the cancerous epithelial cells in the samples and was predominantly cytoplasmic. As demonstrated by PCR, AGR2 expression was significantly associated with positive oestrogen receptor (OR) status but with a negative epidermal growth factor receptor (EGFR) status. These authors also demonstrate that AGR2 is strongly expressed in PCa, another hormone-sensitive tumour. AGR2 was found to be expressed in 34/42 samples (81%). It must be acknowledged though that this study does not comment on whether AGR2 is expressed in benign breast or prostate tissue. For AGR2 to have a potential role in either breast or prostate carcinogenesis then one might have thought that it's increased expression in malignant when compared with benign tissue might have been sought however this does not appear to be the case. AGR2 has been found to be highly expressed in pancreatic cancer as demonstrated by PCR and IHC. AGR2 expression was 14-fold higher in pancreatic cancer tissue when compared to benign tissue or tissue from chronic pancreatitis (99). IHC demonstrated high expression of AGR2 in 56/57 pancreatic cancer samples (98%) and AGR2 is expressed in the majority of pancreatic cancer cell lines (7 out of 9). There was little or no staining in the benign or chronic pancreatitis samples. AGR2 was also detected in the conditioned media from pancreatic cancer cell lines suggesting that it is actively secreted

by cancerous cells. This may be of use when attempting to diagnose or even screen for pancreatic cancer and it may be that the analysis of pancreatic secretions may be a way by which this disease can be detected earlier in selected individuals where the disease overall has a pretty poor prognosis. A review article by Brychtova et al., in 2011 outlines the vast numbers of tissues to date that are known to express AGR2 (100). It suggests that AGR2 is strongly expressed in tissues containing mucus secreting cells and/or those that function as endocrine organs e.g. lung, stomach, colon and prostate. AGR2 is thought to confer a metastatic phenotype and, as much of the mortality from carcinoma in general is brought about by metastasis it therefore may be an important factor in these types of malignancies and as such leads to the possibility of its use as a biomarker in the future.

In 46 matched benign and malignant prostate tissue samples isolated by micro-dissection, AGR2 mRNA expression (relative to GAPDH) was higher ($\geq$ 1.5 fold increase) in 34/46 malignant prostate samples (76%) (101). In 22 samples (48%) there was a > 2 fold increase in the AGR2 mRNA expression ratio in the malignant versus benign prostate tissue samples. An AGR2 staining score, in a smaller number of samples was also significantly higher, according to IHC when comparing benign tissue (n = 12, score = 1.33) with malignant tissue (n = 11, score = 2.45) and high grade PIN (n = 5, score = 2.80). Zhang et al., confirmed the presence of AGR2 protein expression in the benign prostate cell line: PNT-2, and in the highly malignant cell lines: PC3 and PC3M (102). AGR2 expression was not detected in the weakly malignant cell line: LNCaP nor in the highly malignant cell line: DU145. The reasons for this difference of expression are not clear but could be relevant to my data and will be discussed later. AGR2 protein expression appeared to be much higher in the two PC3 cell lines when compared to the benign cell line although it is not clear whether this is statistically significant. This study again, confirmed by IHC that in archived prostate cancer specimens AGR2 stained significantly stronger when compared with BPH tissue. In 7 normal prostate tissue samples the majority stained weakly (n = 5, 71.4%), in 34 BPH cases just over half (n = 18, 52.9%) stained weakly whereas in 65 malignant cases 19 (29.2%)

stained *moderately* positive, and 29 (44.9%) stained *strongly* positive. This increase in strength of staining was significantly higher when comparing malignant to BPH and normal samples. This study suggested that increased AGR2 expression is associated with reduced patient-survival time and thus leads to the possibility of AGR2 being used as a prognostic marker in prostate cancer in the future.

Metastasis is a common cause of morbidity and mortality in prostate cancer and deregulation of AGR2 appears to confer a metastatic phenotype (103). It is postulated by these authors that the expression of AGR2 may in some way be controlled by ErbB3 binding protein 1 (EBP1), a separate molecule that regulates cell growth, apoptosis and differentiation (104). Two LNCaP sub lines (C81 and C4-2B) were used for these expression studies after they had been manipulated to become androgen independent and therefore replicating a more aggressive, metastatic phenotype. Expression of AGR2 (when compared to actin) was higher in the PC3, C81 and C4-2B cell lines as well as the androgen-refractory cell 22Rv1. In the LNCaP cell line there was no increase of AGR2 expression compared with actin. With regards to EBP1 expression in the more aggressive PCa cell lines (PC3 and C81) this appeared to be reduced when compared to actin. With an apparent reciprocal expression pattern with regards to AGR2 and EBP1 it could be possible that EBP1 is a negative regulator of AGR2 expression and therefore its deregulation may lead to a metastatic phenotype. In LNCaP cell lines forced to over-express AGR2 there was found to be a significant increase in motility (Boyden chamber assay) and invasiveness (Matrigel-membrane assay) compared to normal LNCaP cells, whereas silencing of AGR2 in the LNCaP-derived cell line C4-2B blocked invasive behaviour. Metastatic C4-2B PCa cells that were transfected by EBP1 (C4-2BE) expressed lower levels of AGR2 and showed significantly lower rates of migration and invasion as assessed by the above methods. Again this data suggests that metastasis and aggressiveness in PCa may be mediated *through* AGR2 expression but under the direct control of EBP1.

Bu et al., have demonstrated that AGR2 mRNA is present in the urine of men undergoing prostate biopsies at significantly higher levels in those with prostate cancer than in those without and have also shown increasing levels of AGR2 mRNA in malignant prostate tissue when compared with benign (105). It is noted that two transcripts exist for AGR2: short (AGR2St) and long (AGR2Lt) however both encode for the same processed protein. AGR2 mRNA (short and long) expression was significantly higher in 24 low Gleason score (6) and 26 high Gleason score (8-10) prostate samples when compared to 12 benign prostate samples. Interestingly expression of AGR2St was significantly *higher* in Gleason score 6 compared to Gleason score 8-10 prostate tissue (8.54 vs. 2.29); whereas expression of AGR2Lt was *lower* in Gleason score 6 compared to Gleason score 8-10 prostate tissue. These expression studies appear to reflect the heterogeneity of prostate cancer and represent the differing patterns of expression within even similar types of prostate cancer specimens. As tumours become more aggressive i.e. have a higher Gleason score, then the degree of differentiation reduces and it is possibly more difficult to look for reliable and reproducible genes markers for predicting prognosis. What is not clear from this data is what the level of expression was for Gleason pattern 7 disease. Not having any Gleason 7 disease in the clinical samples that have been reported would be rare according to this author's experience. It is not clear whether this pattern was actively excluded from analyses or whether it simply wasn't encountered. Some data on the level of expression of AGR2 in this type of PCa would have been useful, particularly as these patients upon diagnosis are often borderline for either monitoring or radical treatment. Some published evidence to suggest that a particular patient might better be offered monitoring rather than radical treatment, or vice versa may be a very useful prognostic marker in PCa. In keeping with other published data, using IHC these authors were also able to demonstrate an increased level of expression of AGR2 in PCa and PIN when compared to benign samples. With regards to AGR2St expression in urine it has been shown that expression is significantly higher, according to PCR, in urine sediments of men with PCa (n = 31) compared to those without (n = 29). One point worth mentioning with regards to this data is that expression appeared to be marginally higher

(although not significantly) in the urine of men with Gleason score 5-6 compared to Gleason score 7-9 tumours. This means that AGR2St could possibly be in the future as a urinary biomarker for men with a raised serum PSA undergoing prostate biopsy and may more accurately identify those at risk of prostate cancer, however some important points need to be taken into consideration. A significant proportion of men were excluded (11/40 – benign, 8/39 – malignant) as the amount of AGR2 RNA in those urine samples was too low for analysis. It must also be borne in mind that a negative prostate biopsy does not completely exclude PCa (as the biopsy needle may miss a tumour due to a sampling error). Patients in this study may have been allocated to the benign group but actually have PCa that has simply not been sampled. The difference between the groups may not be as statistically significant as is suggested as the groups may not be clearly distinct. Having said that it is potentially a useful marker for identifying those men who are at an increased risk of PCa (i.e. those who have a raised PSA but previous negative biopsies) and many may find providing a urine test after a prostate exam, preferential to further prostate biopsies. AGR2 protein expression was assessed using WBA with various PCa cell lines and expression appeared to be highest in PC3 cells and lower in LNCaP and Du145 cells in keeping with previous data (101). No expression of AGR2 was demonstrated in the benign prostate cell line: BPH1. 22Rv.1 cells stably transfected with AGR2 protein showed a *reduction* in cell proliferation after 48 hours when compared to vector control clones but an *increase* in cell migration and invasion. Silencing of AGR2 in PC3 PCa cells significantly reduced the migratory and invasive ability of these cells.

Maresh et al., demonstrate that AGR2 expression is significantly higher in PCa tissue for two datasets (malignant samples: n = 66 and 62 respectively) using a prostate TMA compared to matched benign tissue. AGR2 expression as determined by spot level histopathology is also significantly higher in PIN lesions and when compared to histologically normal prostate tissue and BPH and between lymph node metastases and normal prostate tissue and BPH. Using the same method AGR2 expression was also

assessed in terms of Gleason score. It was demonstrated that a Gleason score of 3 or 4 was significantly higher than grade 2 and grade 5. Having stated that much of the morbidity and indeed mortality associated with PCa is due to metastatic disease and that AGR2 appears to confer a metastatic phenotype one may have expected AGR2 expression levels to be highest in the most aggressive grade of prostate cancer: Gleason 5. The fact that AGR2 levels are significantly lower in Gleason pattern 5 PCa is therefore surprising. This may be due to the fact that higher Gleason score tumours are associated with a *poorly* differentiated carcinoma and thus are poorly related to its native tissue or even lower grade PCa tissue and thus expression of expected cancer associated genes or proteins is completely lost. If any molecule is to become clinically useful as a biomarker then its ability to predict disease recurrence and mortality is crucial. Following a RRP AGR2 wasn't able to predict disease recurrence in higher stage PCa (III and IV: ≥ T3).In this group relatively *lower* levels of AGR2 expression were highly predictive of disease recurrence to a significant level (106). For the lower AGR2 expression group median recurrence-free time was 14 months compared to 38.5 months for those with higher AGR2 expression. AGR2 expression did not appear to correlate with other clinic-pathological parameters such as Gleason score, margin status and lymph node status.

The evidence presented suggests a promising, albeit complicated potential role for AGR2 in PCa and I was therefore interested to explore the role that AGR2 plays in obesity-driven PCa and its manipulation by the adipokine chemerin.

6.2 Results

In the PC3 cell line there was an increase in AGR2 mRNA expression after 24 and 36 hours of chemerin treatment. There was a highly significant increase in AGR2 mRNA after 24 hours with only one concentration of chemerin: 1nM ($p < 0.01$) compared to basal (0nM). At 36 hours treatment there was a significant increase in AGR2 mRNA expression at all concentrations of chemerin used (0.1-10nM) ($p < 0.05$) compared to basal (0nM). In the LNCaP cell line there were significant increases demonstrated in AGR2 mRNA expression after 4 hours only. There were statistically significant increases ($p < 0.01$) demonstrated with 0.1 and 1nM of chemerin and highly statistically significant increases ($p < 0.001$) demonstrated with 10 nM chemerin stimulation.

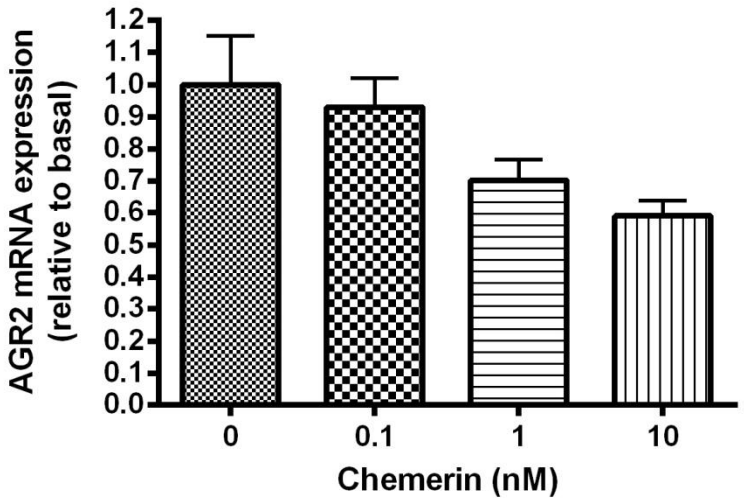


Figure 6-1: Graph demonstrating the effects of chemerin on AGR2 mRNA expression in PC3 cells (n = 3) with respect to basal after 4 hours, (mean + SEM).

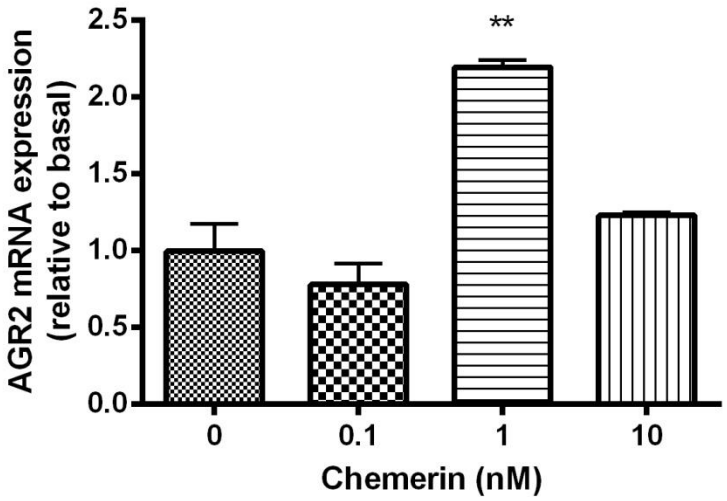


Figure 6-2: Graph demonstrating the effects of chemerin on AGR2 mRNA expression in PC3 cells (n = 3) with respect to basal after 12 hours, (mean + SEM, difference from basal, **: p < 0.01).

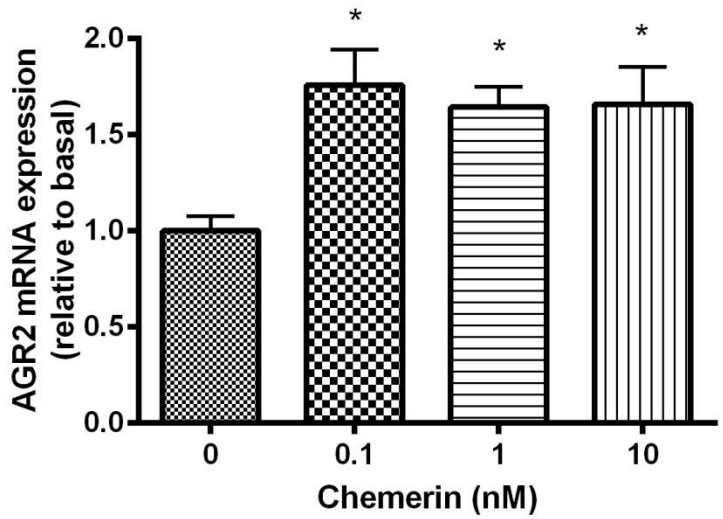


Figure 6-3: Graph demonstrating the effects of chemerin on AGR2 mRNA expression in PC3 cells (n = 3) with respect to basal after 24 hours (mean + SEM, difference from basal, *: p < 0.05).

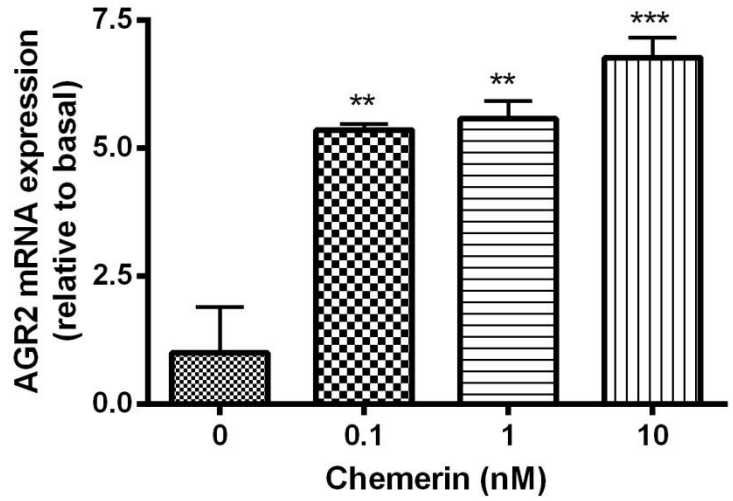


Figure 6-4: Graph demonstrating the effects of chemerin on AGR2 mRNA expression in LNCaP cells (n = 3) with respect to basal after 4 hours, (mean + SEM, difference from basal, **: p < 0.01, ***: p < 0.001).

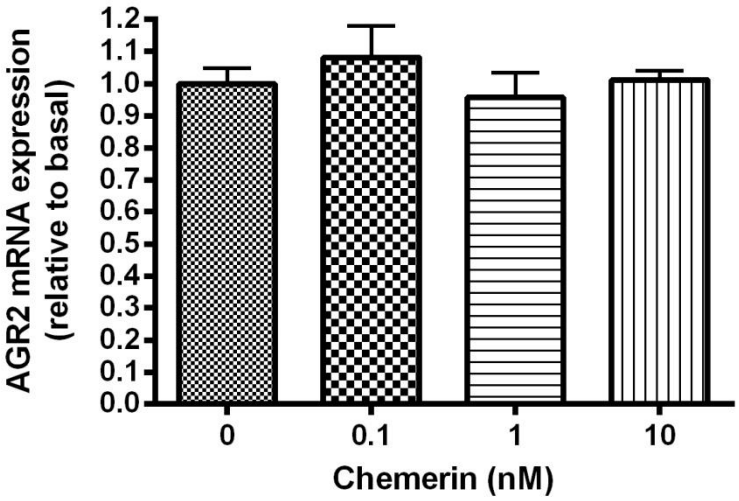


Figure 6-5: Graph demonstrating the effects of chemerin on AGR2 mRNA expression in LNCaP cells (n = 3) with respect to basal after 24 hours (mean + SEM).

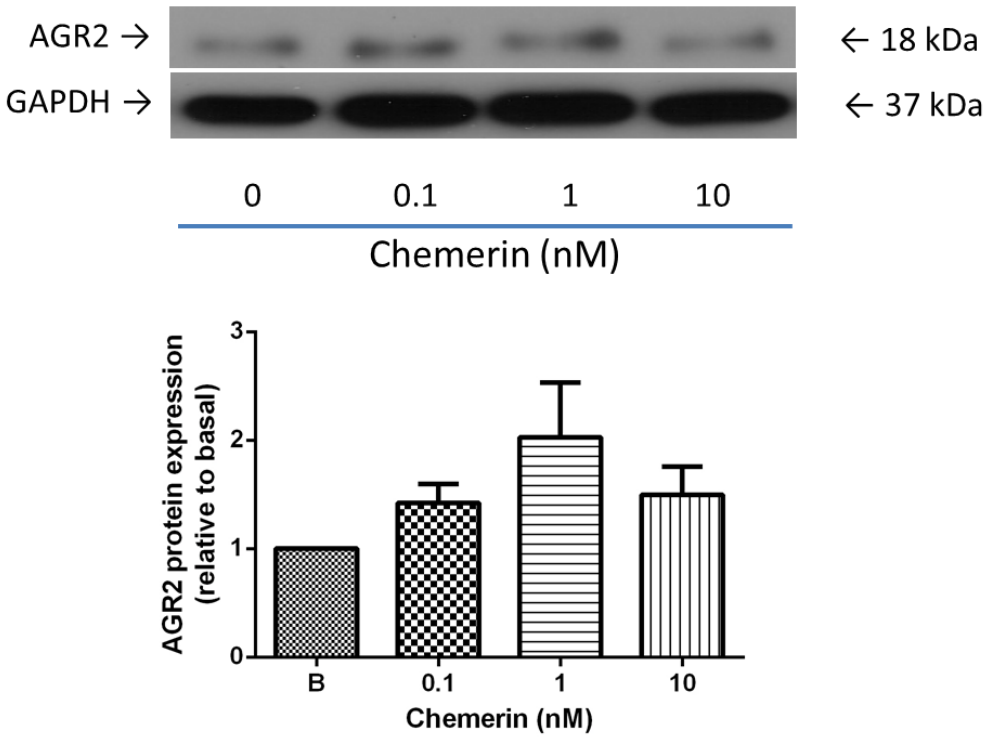


Figure 6-6: Representative western blot (top) and graphic representative (bottom) demonstrating the effects of chemerin on AGR2 protein expression in PC3 cells (n = 3) with respect to basal after 24 hours (mean + SEM).

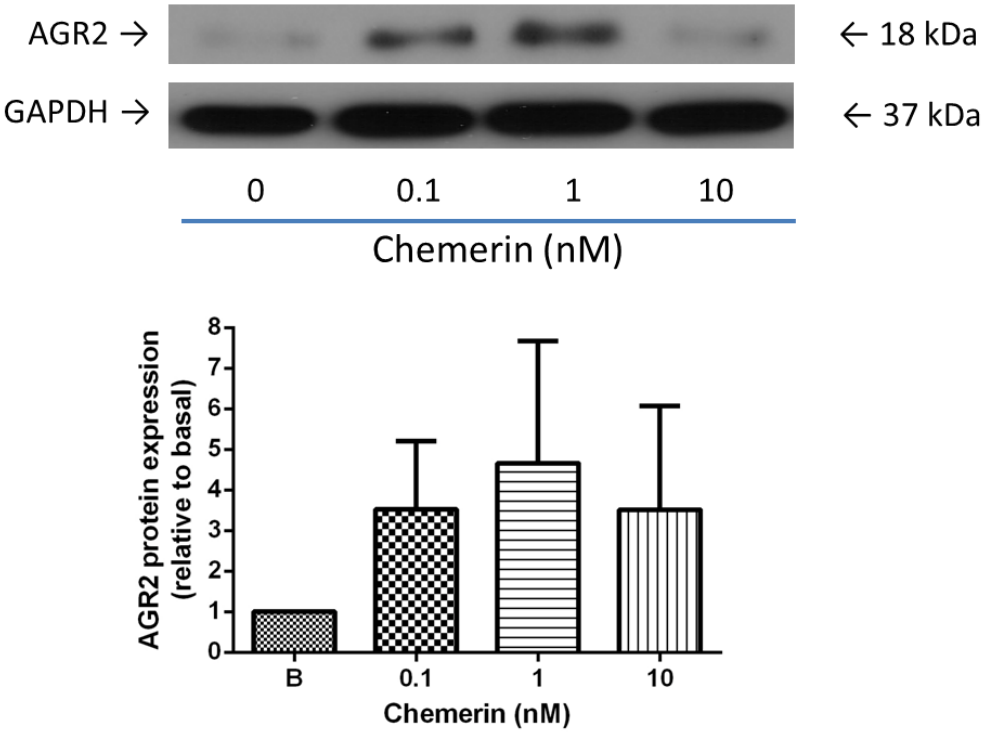


Figure 6-7: Representative western blot (top) and graphic representative (bottom) demonstrating the effects of chemerin on AGR2 protein expression in PC3 cells (n = 3) with respect to basal after 36 hours (mean + SEM).

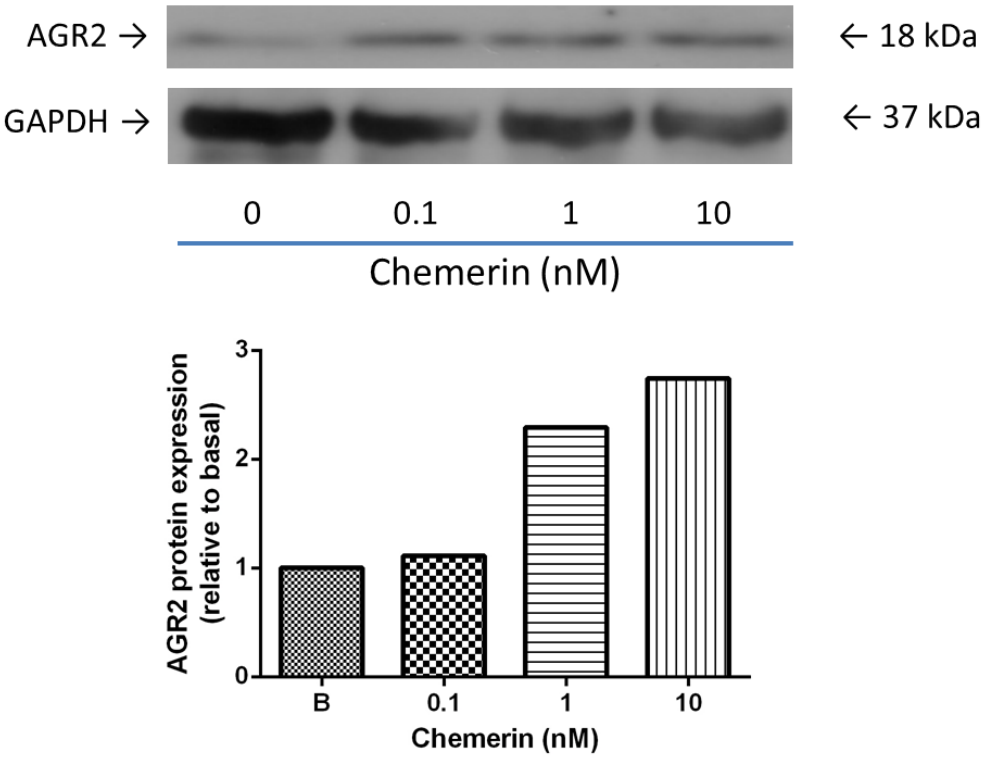


Figure 6-8: Representative western blot (above) and graphic representative (below) demonstrating the effects of chemerin on AGR2 protein expression in LNCaP cells (n = 1) with respect to basal after 24 hours (mean + SEM).

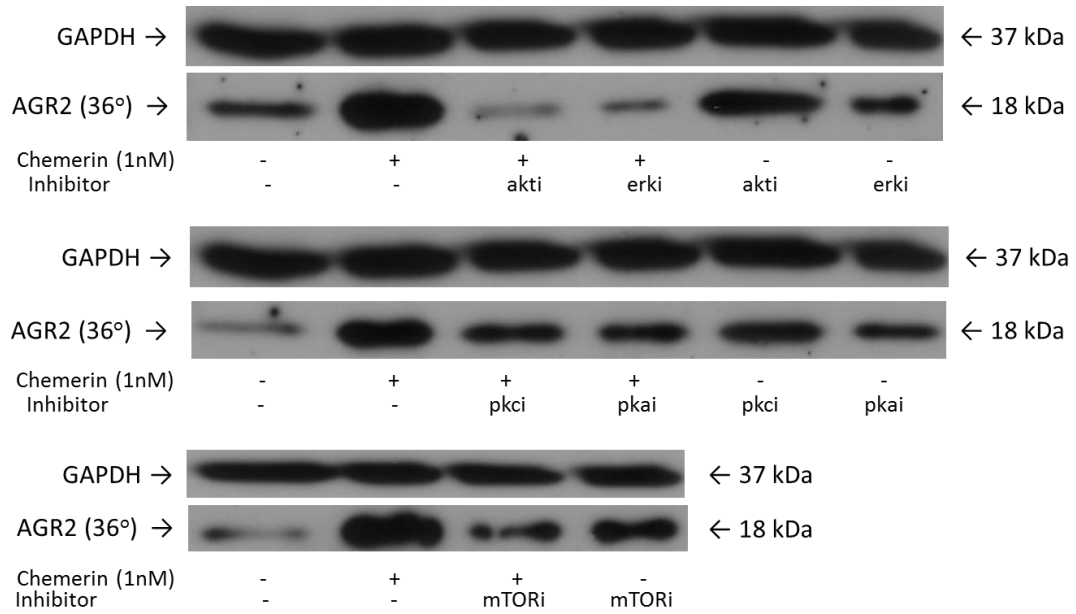


Figure 6-9: Representative western blots demonstrating the effects of various pathway inhibitors (AKT, ERK, PKC, PKA and mTOR) alone and in combination with 0.1nM chemerin on AGR2 protein expression in PC3 cells after 24 hours.

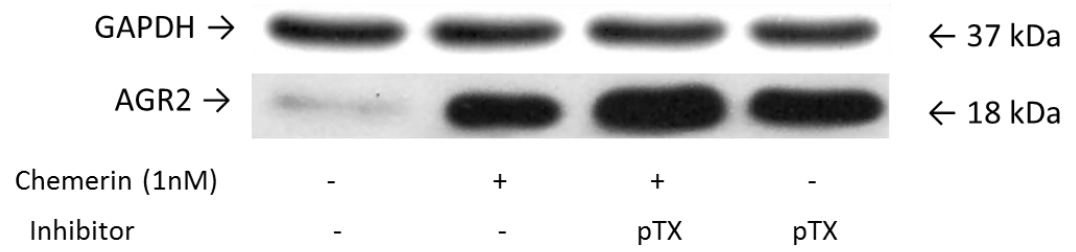


Figure 6-10: Representative western blots demonstrating the effects of pertussis toxin (pTX) +/- chemerin on AGR2 expression in PC3 cells after 24 hours.

In the PC3 cell line there is an increase in AGR2 protein expression with chemerin stimulation compared to basal. The increase appears to be most notable with 1nM chemerin at both time points used (24 and 36 hours), however there appears to be at least some increase with all chemerin concentrations used. This increase appeared to be most dramatic after 36 hours compared to 24 with a six to ten-fold increase in AGR2 protein expression at this time point. The changes in protein expression corroborates with the changes in AGR2 mRNA expression noted after 24 and 36 hours stimulation. The increase in AGR2 mRNA and protein expression was similar after 24 hours as 1nM chemerin showed the highest

increase. After 36 hours there was a significant increase in AGR2 mRNA and protein expression with all concentrations of chemerin: 0.1-10nM. In the LNCaP cell line due to difficulties with cell culture and contamination with this cell line only one time point was used: 24 hours. Unlike in the mRNA expression studies there appeared to be an increase (two to three-fold) in AGR2 protein expression after 24 hours after stimulation with higher concentrations (1-10 nM) of chemerin compared with basal (0nM). When PC3 cells were treated with various inhibitors alone and in combination with chemerin (Figure 6-9), some slightly unusual results were produced. Chemerin treatment alone caused an increase in AGR2 protein expression as previously seen (Figure 6-7). With the inhibitors alone there also appeared to be an *increase* in AGR2 expression in PC3 cells after 36 hours with an apparent *reduction* in AGR2 expression when the AKT & ERK inhibitors were used in combination with chemerin As pertussis toxin (pTX) has been shown to inhibit G-protein coupled receptors (GPCR) in mammalian cells PC3 cells were treated with pTX in combination with 1nM chemerin for 24 hours. pTX leads to the ADP-dependent ribosylation of GTP-binding proteins (107) and this technique was used to test signal transduction in these cells with regards to AGR2 expression. It is clear from the above western blots that pTX appears to increase the expression of AGR2 both dependently or independently of chemerin activation suggesting that chemerin does not appear to be affecting the activation of AGR2 activation through this particular pathway.

6.3 Discussion

The Anterior Gradient 2 (AGR2) gene appears to confer a metastatic phenotype in most adenocarcinomas, including prostate cancer. It is associated with more aggressive disease and thus is a potential prognostic marker that can be used in the future management of patients initially diagnosed with PCa. As metastatic disease is the commonest cause of mortality in PCa, strategies to identify men at risk of developing metastatic disease could identify those patients who would benefit from more aggressive or earlier treatment. The data presented not only demonstrates the increased expression of AGR2 in PC3 cells

compared to LNCaP cells but a potential role of chemerin in the manipulation of AGR2 expression and therefore a role for AGR2 in obesity related prostate cancer. It has been demonstrated that knockdown of AGR2 induces a state of cellular senescence in prostate cancer cells (108). Senescence occurs normally after approximately 50 divisions in vitro and describes a state in which a cell has lost its ability to divide. AGR2 appears to prevent a cell becoming senescent and therefore may mean that it has a crucial role to play in carcinogenesis. AGR2 is secreted into the urine from prostate cells and may be useful as marker of prostate cancer in men prior to prostate biopsy or even after a negative prostate biopsy or biopsies in whom the risk of prostate cancer is still thought to be high e.g. those with an abnormal prostate examination or a persistently raised PSA. Patients generally prefer non-invasive over invasive investigations and may find a urine test more preferable than repeated blood tests or prostate biopsies, which some men in particular find uncomfortable or painful. The development of an ELISA to detect AGR2 in urine (109) is a promising step forward and although not currently clinically applicable may be a promising test for prostate cancer diagnosis in the future. There is also a possibility that urine AGR2 levels could either be used as a risk stratifying tool in men at risk of prostate cancer i.e. those with a raised PSA with previous negative biopsies or with a strong family history. AGR2 has also been isolated from the peripheral blood of patients with prostate cancer (110). In patients with metastatic prostate cancer AGR2 levels are elevated at the mRNA and protein level. This is particularly clinically relevant as some patients with non PSA-secreting or anaplastic metastatic PCa may have very advanced disease but with very low levels of PSA (< 1ng/ml).This means that it may not be possible to simply rely on PSA levels to assess the activity of the disease and to plan further treatment or response to treatment.

The western blot data presented above demonstrates some interesting findings with regards to AGR2 expression in PC3 cells and potentially leads to some information with regards to how AGR2 expression is mediated. All the blots demonstrate increased expression of the AGR2 protein with regards to basal after treatment with 1nM chemerin after 24 hours. With

regards to the AKT and ERK inhibitors (and to a certain extent the mTOR inhibitor) there appeared to be a reduction in AGR2 expression after treatment of PC3 cells in combination with chemerin after 24 hours. Indeed with AKT and ERK inhibitors AGR2 protein expression was reduced to less than basal levels after treatment in combination with chemerin. Treatment with the AKT/ERK inhibitors alone demonstrated AGR2 expression that appeared to be at, or just below AGR2 expression after treatment with 1nM chemerin alone. In the previous chapter (5.6) AKT/ERK activation was stimulated by chemerin and it seems to be that inhibition of this pathway (synchronously activated by the adipokine chemerin) leads to the reduction in activation of the pro-metastatic gene AGR2. Potentially therefore, activation of the AKT and ERK pathways not only appears to be the mechanism by which increased cellular proliferation is noticed (in PC3 cells at least) but also a potential mechanism behind why there is an increased expression of AGR2 through chemerin treatment. It must be noted that this difference has only been demonstrated in the more aggressive PCa cell line (PC3) and not the less aggressive cell line (LNCaP). It is therefore difficult to expect that without proof of AKT/ERK activation in the LNCaP cell line that chemerin is solely responsible for the increased expression of AGR2 seen in these cells. As AGR2 is a pro-metastatic gene and LNCaP cells have limited metastatic potential then this is perhaps at the same time unsurprising. Given the fact that increased AGR2 expression is demonstrated with the various inhibitors alone, clearly AGR2 expression is complex and under the control of multiple pathways and mechanism and further work is needed to clarify its behaviour in PCa In the PC3 cells it is perhaps to be expected that expression of the pro-metastatic gene (AGR2) is increased (in a cell line with a higher metastatic potential) after stimulation with chemerin and potentially explains in part, the increased mortality demonstrated in obese men diagnosed with PCa.

7 The role of serum chemerin levels in adult men with prostate cancer

7.1 Serum collection

Blood samples were collected from patients enrolled in the study, on the day of surgery and serum was extracted. Every effort was made to collect blood samples in the fasting state and prior to midday; the time at which the blood was taken was noted. Serum was collected using 5ml Vacutainer® SST™ (serum separator tubes) blood collection tubes (BD, Franklin Lakes, NJ USA). Samples were centrifuged at 3000 rpm for 10 minutes with maximum acceleration and braking. The serum was carefully aspirated from the rest of the blood constituents and pipetted into cryo-vials. Samples were stored at -80^{o}C and were thawed slowly on ice when needed. A body composition monitor (Tanita BC-601, Arlington Heights, IL, USA) was used to measure patient's weight, percentage body mass, muscle and bone mass and percentage body water. Height was measured in a standardised fashion when patients attended a pre-operative assessment clinic prior to the day of surgery. BMI was later calculated from the patient's height and weight using the approved formula. The body scanner uses bioelectric impedance analysis (BIA) to measure the above parameters in a reproducible and standardised way. Serum chemerin levels were calculated using ELISA kit as described earlier on serum samples diluted to 1:8 with reagent diluent. Patients were categorised as to having either benign or malignant prostate disease according to histology taken at the time of entry into the study, a prior clinical or histological diagnosis of prostate cancer or a subsequent histological diagnosis of prostate cancer *after* inclusion in the study.

7.2 Results

A total of 88 men were recruited to the study between Dec 2010 and March 2012 with a mean age of 71.8 years (52.5 – 91.3).

	Benign	n	Malignant	n	Significance
Age (Years)	73.3	49	70.0	39	NS (p = 0.081)
BMI (kg/m^2)	28.4	49	27.5	37	0.274
Body Fat (%)	27.4	37	26.8	31	0.653
Muscle Mass (Kg)	57.2	37	55.9	31	0.546
Bone Mass (Kg)	3.0	37	3.0	31	0.683
Daily Calorie Intake (kcal)	3075	37	3051	31	0.854
Metabolic age (years)	68.9	37	68.2	31	0.820
Body Water (%)	52.3	37	52.6	31	0.808
Visceral fat rating	17	37	15	31	0.224
Chemerin (ng/ml)	4.42	47	4.05	36	**0.058**
Glucose (mmol/l)	5.9	37	5.7	31	0.679
Cholesterol (mmol/l)	4.7	44	5.1	35	0.087
HDL (mmol/l)	1.3	44	1.5	35	**0.026**
PSA (ng/ml)	8.2	47	13.0	34	0.198
Testosterone (nmol/l)	12.3	43	11.0	32	0.291
Sex Hormone Binding Globulin (nmol/l)	54.2	14	49.6	20	0.697
Creatinine (micro-mol/l)	103.1	47	91.4	35	0.130
Corrected calcium (mmol/l)	2.20	46	2.21	34	0.313
Alkaline Phosphatase	78.3	47	72.6	34	0.279

Table 7-1: Table demonstrating differences in the mean of physical and chemical characteristics between the two groups (benign and malignant) NS = not significant.

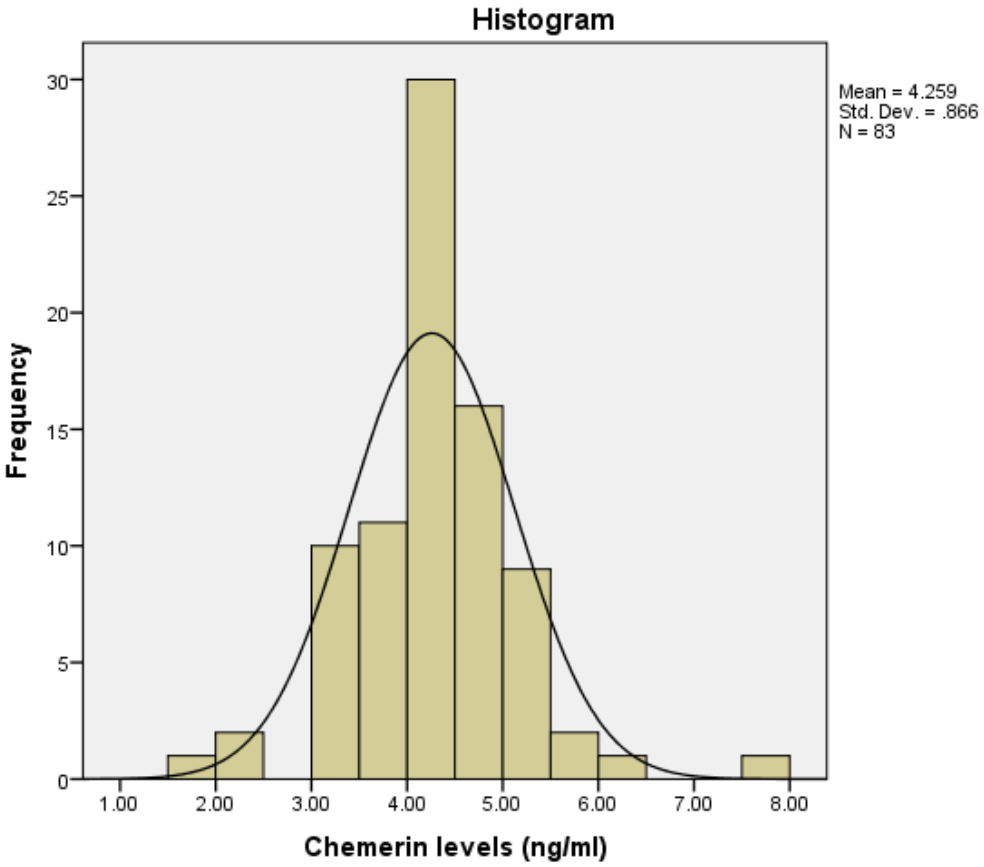


Figure 7-1: Histogram demonstrating frequency distribution of chemerin levels (ng/ml) for patients recruited.

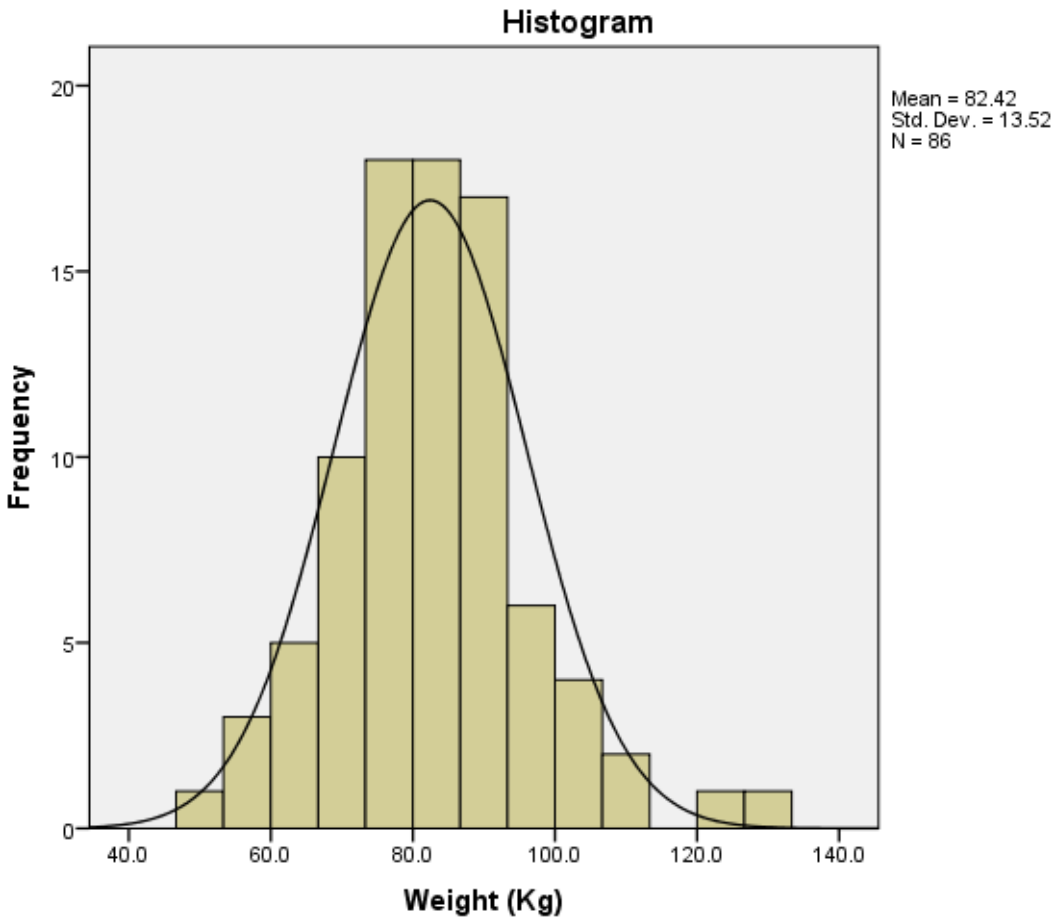


Figure 7-2: Histogram demonstrating frequency distribution of weight (Kg) for patients recruited.

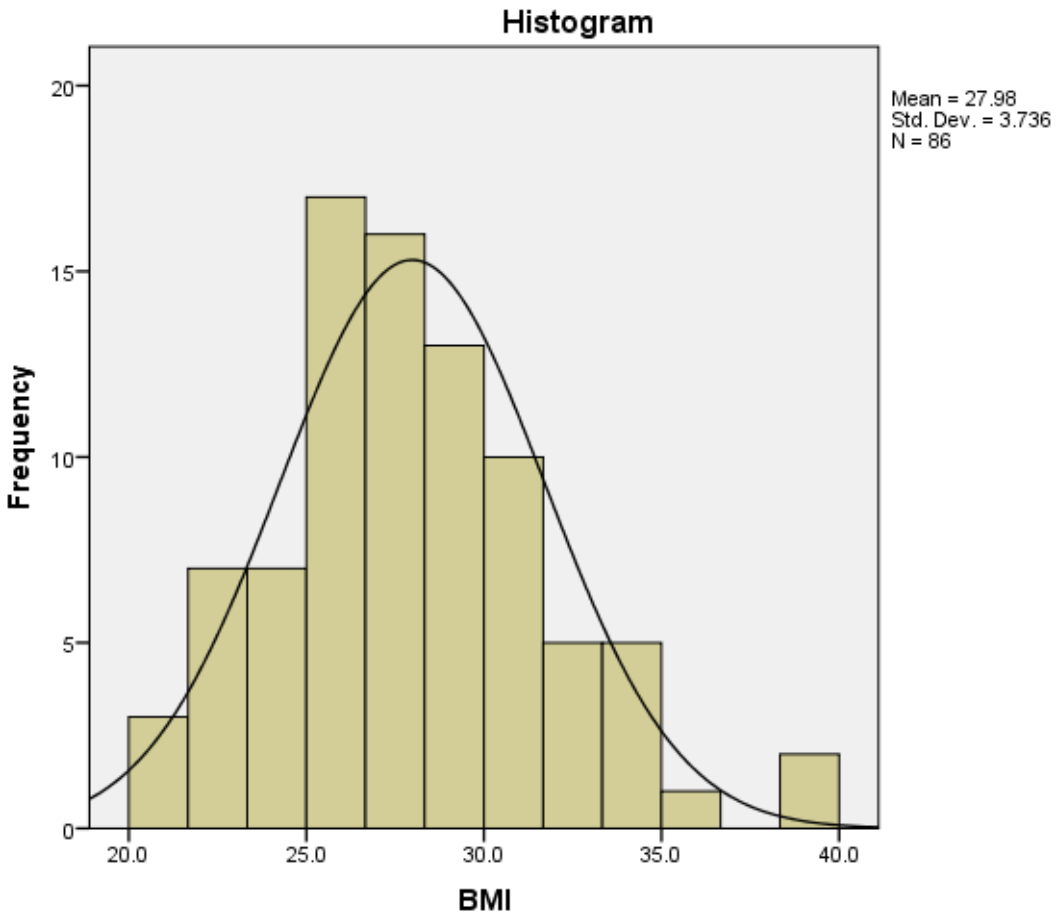


Figure 7-3: Histogram demonstrating frequency distribution of Body Mass Index (Kg/m^2) for patients recruited.

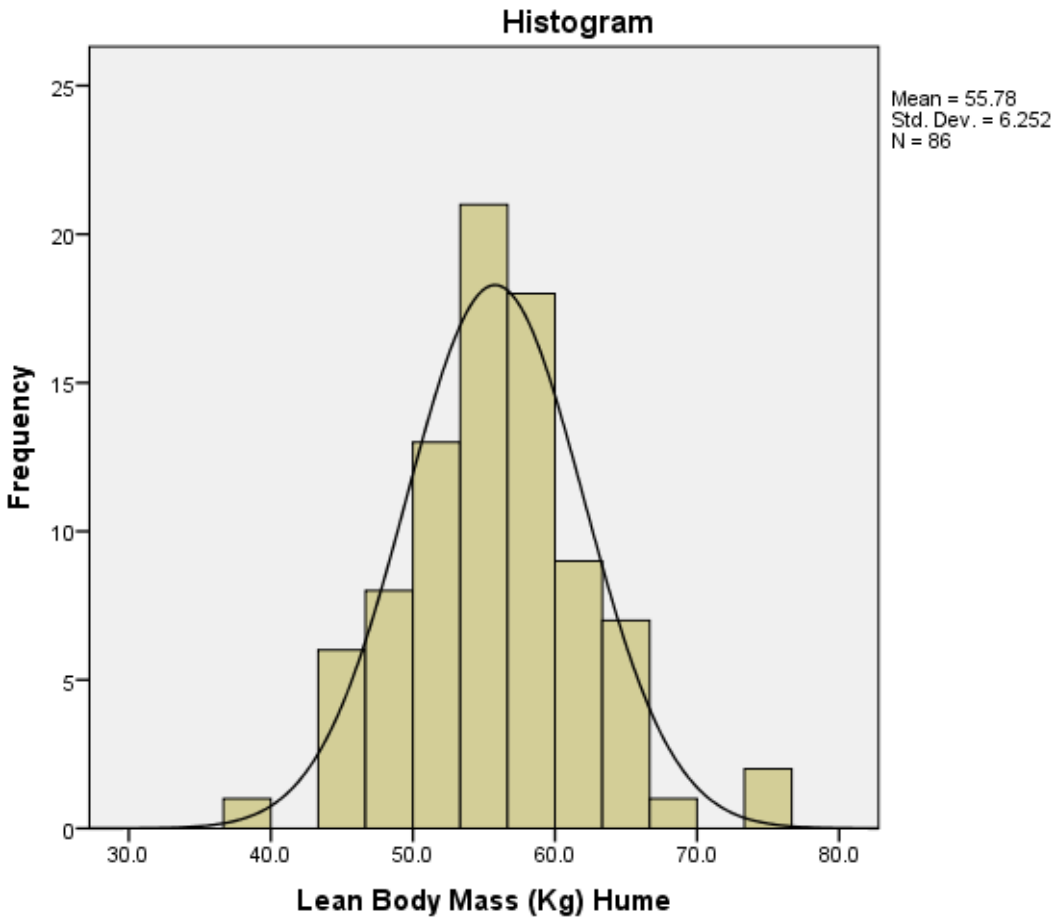


Figure 7-4: Histogram demonstrating frequency distribution of lean body mass (Kg) for patients recruited.

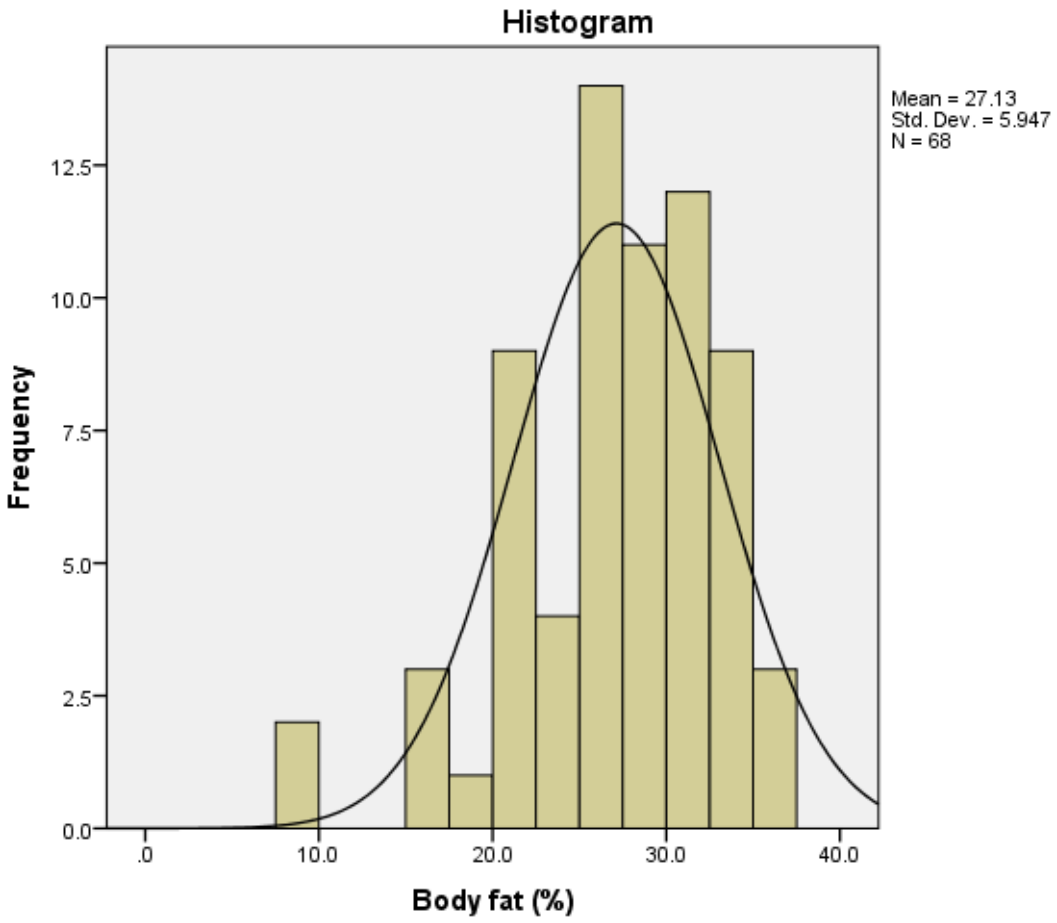


Figure 7-5: Histogram demonstrating frequency distribution of body fat (%) for patients recruited.

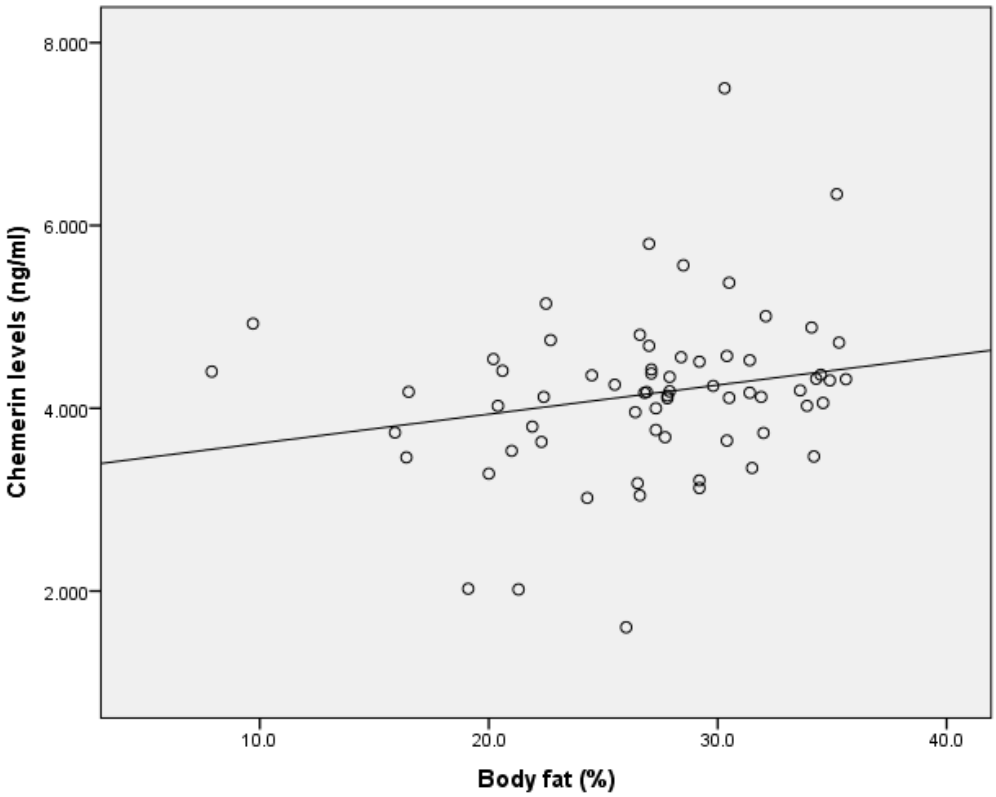


Figure 7-6: Scatterplot demonstrating the relationship between serum chemerin levels (ng/ml) and body fat (%).

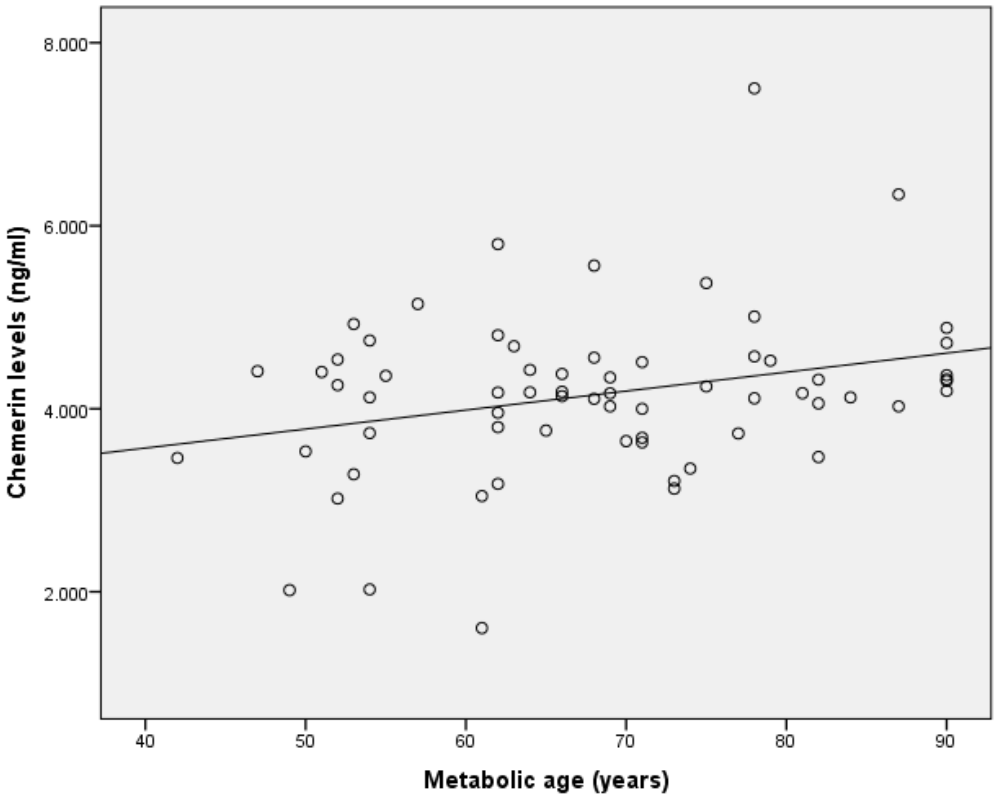


Figure 7-7: Scatterplot demonstrating the relationship between chemerin levels (ng/ml) and metabolic age (years).

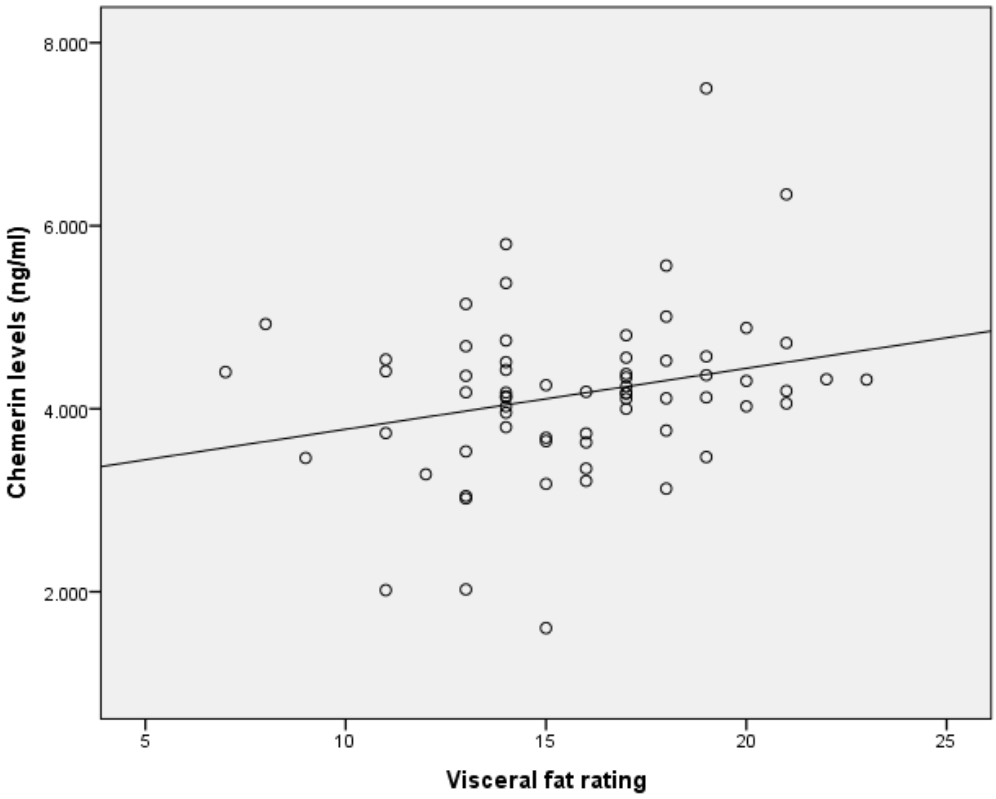


Figure 7-8: Scatterplot demonstrating the relationship between chemerin levels (ng/ml) and visceral fat rating.

Correlation of chemerin (ng/ml) with:	Pearson correlation coefficient	Significance (2-tailed)	N
Weight	0.036	0.745	83
BMI	0.191	0.084	83
Body fat	**0.210**	0.091	66
Lean body mass (Hume)	-0.068	0.541	83
Muscle mass	-0.166	0.183	66
Bone mass	-0.145	0.247	66
Daily calorie intake	-0.154	0.217	66
Metabolic age	**0.289**	**0.019***	66
Body water	-0.155	0.214	66
Visceral fat rating©	**0.249**	**0.044***	66

Table 7-2: Table demonstrating Pearson correlation scores (PCC), level of significance and n number for correlation of chemerin and multiple body parameters. * = p < 0.05.

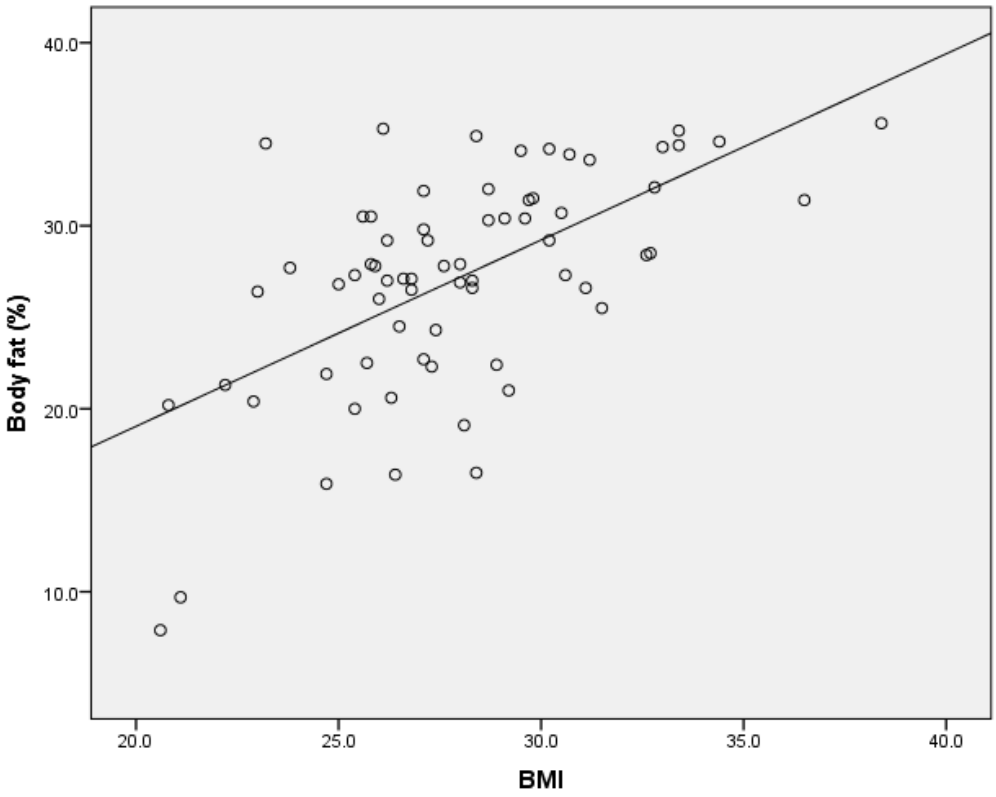


Figure 7-9: Scatterplot demonstrating correlation between BMI and body fat (%).

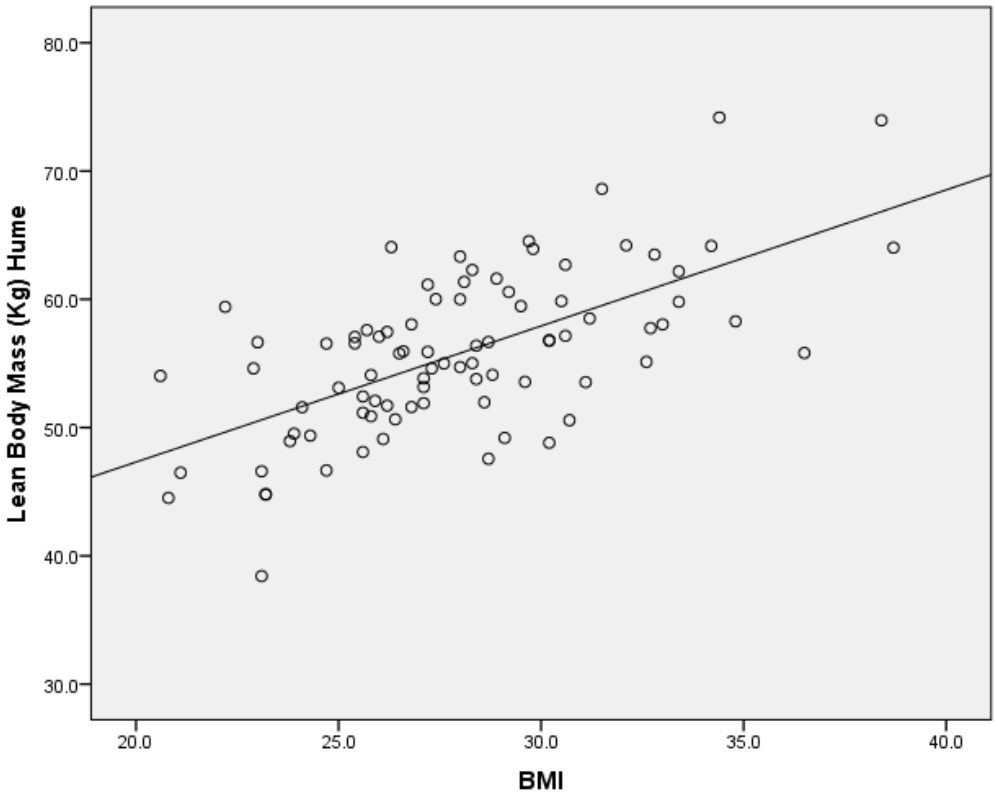


Figure 7-10: Scatterplot demonstrating correlation between BMI and lean body mass (Kg).

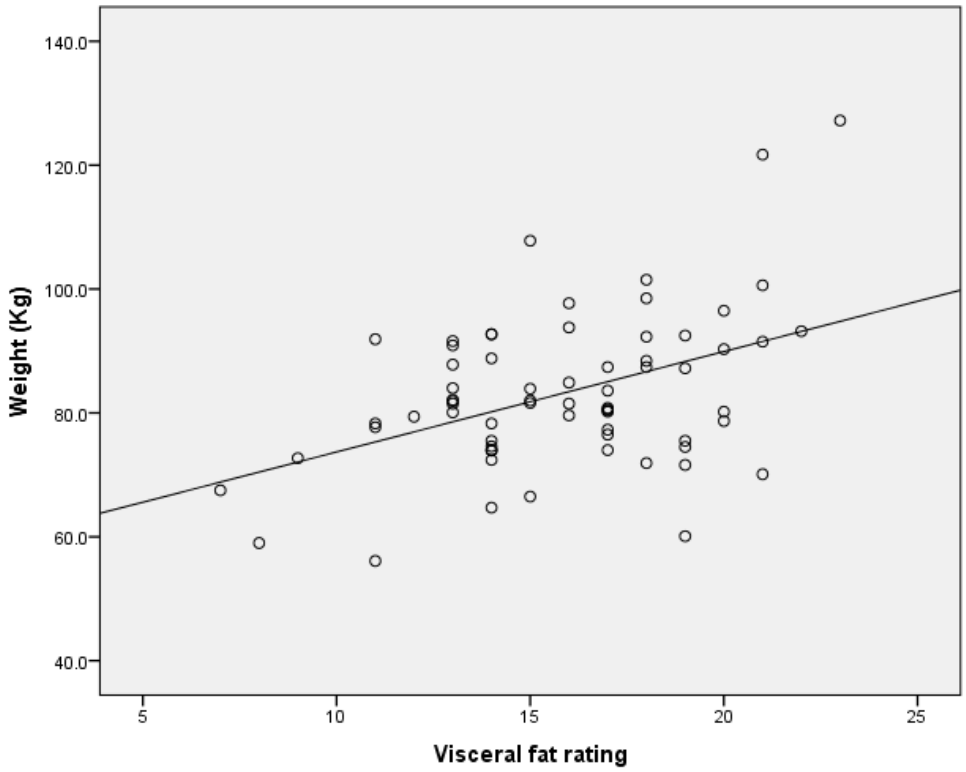


Figure 7-11: Scatterplot demonstrating correlation between visceral fat rating™ and weight (Kg).

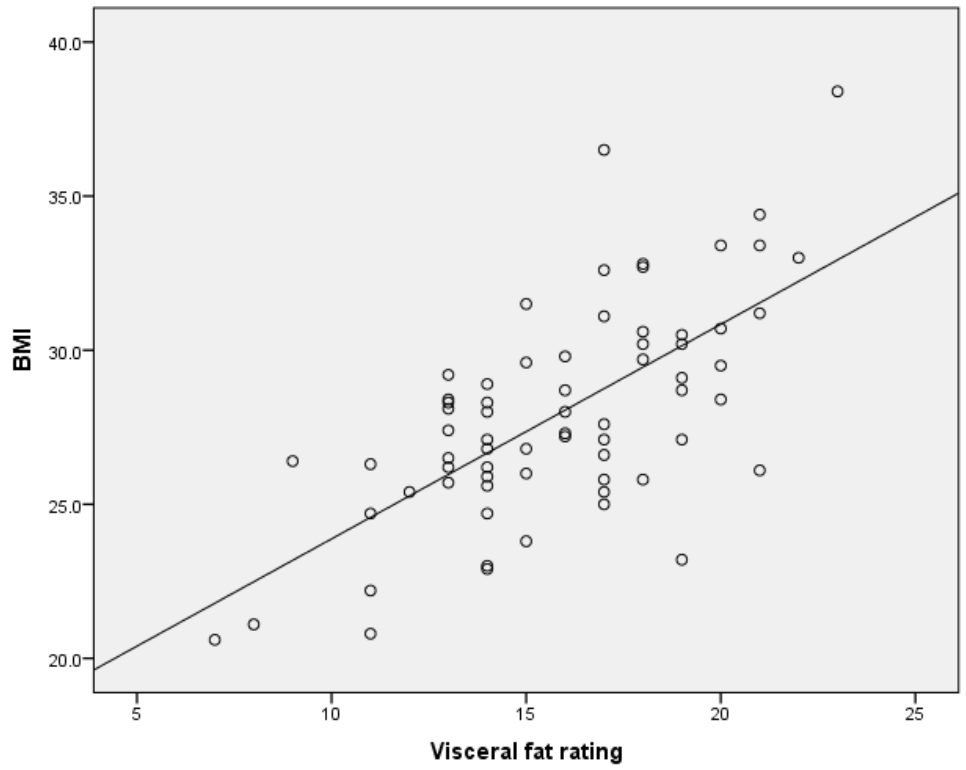


Figure 7-12: Scatterplot demonstrating correlation between visceral fat rating™ and BMI (Kg/m^2).

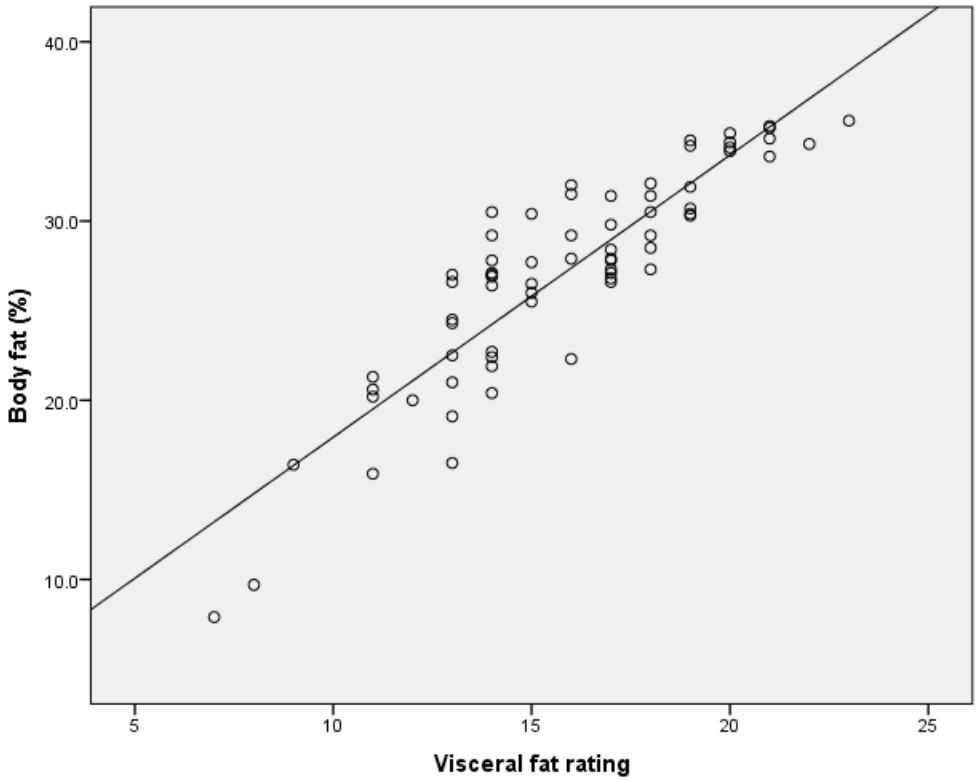


Figure 7-13: Scatterplot demonstrating correlation between visceral fat rating™ and body fat (%).

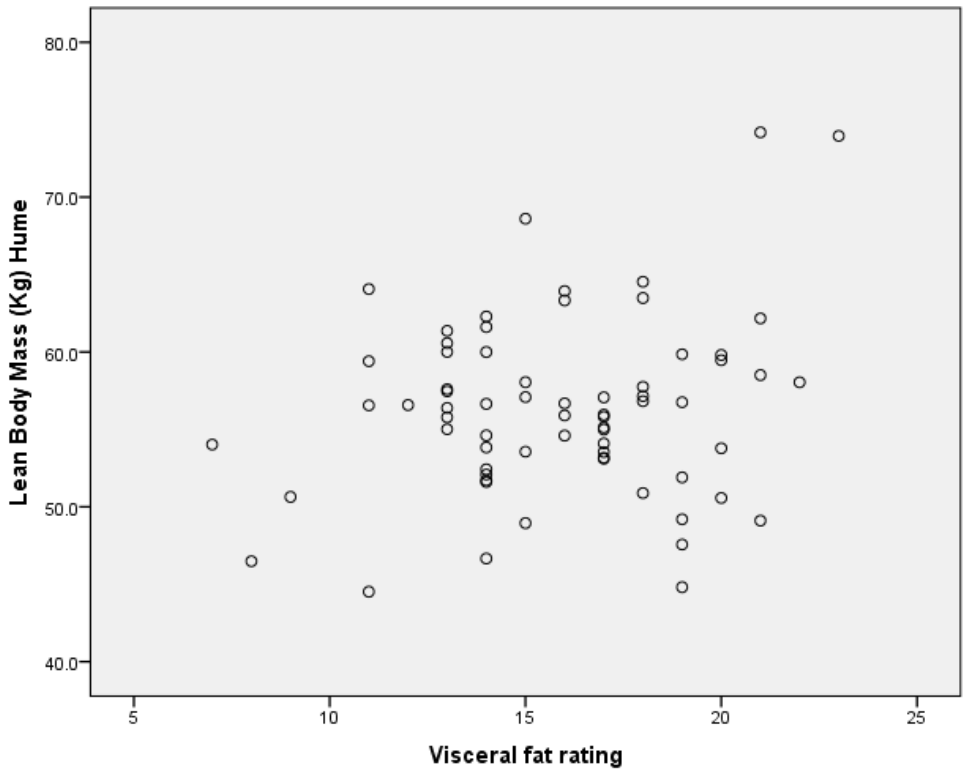


Figure 7-14: Scatterplot demonstrating correlation between visceral fat rating™ and lean body mass (Kg).

Correlation of visceral fat rating© with	Pearson correlation	Significance (2-tailed)	N
Weight (Kg)	**0.437**	**P < 0.01**	68
BMI (Kg/m^2)	**0.669**	**P < 0.01**	68
Body fat (%)	**0.895**	**P < 0.01**	68
Lean Body Mass (Kg)	0.211	P = 0.084	68

Table 7-3: Table demonstrating Pearson correlation score (PCC), level of significance and n number for correlation between visceral fat rating™ and multiple body parameters.

7.3 Discussion

The two groups (benign and malignant) are comparable in virtually all physical and chemical characteristics (according to the mean values) apart from HDL levels (1.3 vs 1.5mmol/l, p = 0.026), see Table 7-1. HDL levels were significantly higher in those men with proven PCa. It is not clear what the reasons for these elevated HDL levels are. This is in contradiction to previously published data as HDL has been shown to be *inversely* associated with PCa risk. In a large cohort of men (n = 69 735) aged over 35 at baseline, those with lower HDL levels at enrolment were noted to be at an *increased* risk of developing PCa (111). During this study 2 008 cases of PCa were identified. Mondul et al., demonstrated in another large cohort (n = 29 093) that higher HDL levels were associated with a decreased risk of PCa. It was also noted during this study that higher cholesterol levels are also associated with an increased risk of overall and advanced PCa (112). In the small cohort of patients in this study, (n = 88) it can be seen that the mean cholesterol level was higher in the malignant group compared with the benign group, however this failed to reached a significant level (4.7vs 5.1mmol/l, p = 0.087).

It can be noted that the chemerin levels in the benign group were *higher* (4.42ng/ml) than in the malignant group (4.05ng/ml). As described above this difference did not reach statistical significance but the p value (0.058) is close to a significant result. It may have been expected that if chemerin plays a role in the pathogenesis of prostate carcinogenesis (i.e. the development of malignant cells from benign prostate cells) then one may well have expected

significantly higher levels of chemerin in the malignant group. It may be the case however, that the higher chemerin levels that exist in patients with benign prostate disease leads to favourable conditions for the development of prostate cancer. The results from the serum sample are in keeping with the data presented from the mRNA/protein tissue levels (chapters 3.2 and 3.3) in that higher levels of chemerin (albeit not significant) were found in benign prostatic tissue. After the development of PCa the higher amount of chemerin may reduce as PCa cells are poorly differentiated and do not closely resemble native prostate tissue. The amount of chemerin produced may reduce therefore as PCa develops resulting in lower serum chemerin levels.

The histogram of chemerin levels (Figure 7-1) demonstrates the distribution of serum chemerin levels in adult men with prostate disease. The chemerin levels appear to be normally distributed with a mean chemerin level of 4.26ng/ml, the SD being 0.87. Having proven that the chemerin levels appear to be normally distributed in both groups of men (benign and malignant), I was able to apply common statistical analyses on this data, such as a student's t-test when looking for significant values.

The above histograms describe the frequencies and distribution of weight (Kg, Figure 7-2), BMI (Kg/m^2, Figure 7-3), lean body mass (LBM, Kg, Figure 7-4) and body fat (%, Figure 7-5) in the cohort of participants. All of the parameters appear to be normally distributed apart from a few outliers in each group. In order for meaningful comparisons to be drawn between the two groups (malignant and benign) it is important to see whether the data obtained is normally distributed before statistical tests such a student's t-test can be performed. It is interesting to note that the mean BMI for all participants is $27.98Kg/m^2$ which is "overweight", as defined by the world health organisation (WHO). It is important to note that the number of participants included in this study is low and may not be an entirely accurate reflection of the general population but it does seem to suggest an increasing problem of increasing weight and obesity in the local, and possibly UK population. It can be seen from Table 7-1 that the mean BMI and % body fat in the benign

group is higher (albeit not significantly) than in the malignant group (28.4Kg/m^2 vs 27.5 and 27.4% vs 26.8). If obesity were to be clearly associated with PCa then you it may have been expected for BMI or % body fat to be higher in the malignant group rather than the benign group. As mentioned in the introductory chapter (1), obesity is associated with BPE (13). Men who go on to develop BPE are more likely to go on to develop symptoms of bladder outflow obstruction and need treatment. Men who have symptomatic bladder outflow obstruction will often require medical treatment for their condition but a small proportion will ultimately require surgical treatment if their symptoms worsen despite the medical therapy. The most common operation performed for enlargement of the prostate is a TURP and a large proportion of men in the benign group had this operation. The higher BMI seen in the benign group may therefore be a reflection on those having surgery for prostate enlargement rather then there being any association with PCa risk. The number of patients in either cohort is probably too small to expect to find a difference between the two groups unless the difference is very pronounced which it appears it isn't.

As can be seen from the scatterplots above there appears to be a *positive* correlation between serum chemerin levels and percentage body fat (Figure 7-6), metabolic age (Figure 7-7) and visceral fat rating (Figure 7-8). No statistically significant correlation was demonstrated between chemerin levels and weight, BMI, lean body mass, muscle mass, bone mass, daily calorie intake or body water (see Appendix C). Although there is no statistically significant correlation between chemerin levels and BMI or body fat there appears to be a positive association between these variables and according to the Pearson correlation coefficient (PCC) (Table 7-2) there is a *small* correlation between these variables. As discussed in chapter 4, full length human chemerin causes increased cell proliferation, migration and reduces apoptosis in the human PCa cell lines PC3 and LNCaP. In interpreting this data, it is important to remember that this is *in vitro* and conducted in artificial circumstances; however it certainly suggests that, as an adipokine, chemerin is found at increasing levels in individuals with a higher BMI and % body fat. It is interesting to note that if chemerin levels

are positively associated with increasing BMI and body fat levels then obese individuals (who are suspected as having an increased risk of PCa) might have an increased incidence or risk of PCa possibly mediated via the action of the adipokine chemerin. As far as this author is aware this is the only discussion on the role of chemerin in PCa and this is therefore an exciting finding and certainly warrants further exploration. The use of a blood test to measure a person's chemerin levels could potentially be used, in conjunction with a person's BMI, family history, PSA level and prostate examination as a marker for risk of developing the disease and could be used to risk stratify a patient or guide treatment for that patient.

BMI is a commonly used marker of obesity and is probably the most widely used and reproducible measure of obesity in the literature and approved by the WHO. One of the drawbacks with using BMI to estimate the level of obesity is that it does not take into account the person's body shape and it makes no differentiation between muscle and fat mass. It must be acknowledged however that BMI is easy to calculate and is reproducible making it simple and easy to use in studies investigating body size, weight and obesity. Generally a higher BMI is associated with a higher percentage body fat and that has been confirmed in my own set of participants (BMI vs % Body fat, PCC: 0.602, $p < 0.01$, Figure 7-9). One has to accept that although BMI is not a direct measure of body fat but there appears to be a significant correlation between the two. Inferring that a rise in BMI leads to a rise in body fat seems to be a safe assumption to make based on the data presented here. Using the following formula devised by Hume I was able to calculate lean body mass (total body mass minus total fat mass): $LBM = (0.32810 \times Weight) + (0.33929 \times Height) - 29.5336$(113). Using this formula a strong correlation was also demonstrated between BMI and LBM (PCC: 0.634, $p < 0.01$, Figure 7-10). This data demonstrates that when comparing BMI with percentage body fat and LBM all are positively correlated with one another. It must be noted that the calculations performed by Hume were based on the measurements of only 29 males. It is also important to note that the accepted body fat percentage differs

between sexes and that different correction factors are applied depending on the gender of the participant.

There is a statistically significant positive correlation between chemerin levels and metabolic age (PCC: 0.289, p = 0.019, Figure 7-7). As far as this author is aware no previous correlation between chemerin levels and age or metabolic age has been reported in the literature previously. Metabolic age is calculated by calculating one's basal metabolic rate (BMR) and comparing it to the expected BMR for one's chronological age (years). BMR was obtained by the calibrated body composition monitor and is dependent upon: actual age, weight, height, activity level, body fat mass and lean body mass. Unfortunately the exact formula by which metabolic age was calculated in this cohort is unavailable to the author as it is protected by a company patent (Tanita). It is therefore impossible to comment on the validity of this parameter but the relationship of chemerin to metabolic age is an interesting one. The higher chemerin levels seen in increasing metabolic years poses the possibility that this may play a part in the increased incidence of PCa seen in older men. It may be that the increasing serum chemerin levels leads to a cumulative increased risk of prostatic malignancy for metabolically rather than simply chronologically older men.

Chemerin levels are positively correlated with visceral fat rating™ (PCC: 0.249, p = 0.044, Figure 7-8). The visceral fat rating™ is a measure of the amount of visceral fat present in that particular person. The visceral fat rating™ is determined by the body composition analyser and varies from 1-59. The higher the visceral fat rating™ the greater the amount of visceral fat that particular patient has. It is thought that visceral fat is more important in terms of a person's body fat rather than peripheral or subcutaneous fat. As previously mentioned (chapter 1) it is thought that visceral fat may be associated with an increased risk of PCa. It is thought to be that the local action of adipokines secreted from visceral fat directly stimulates the development of PCa. Unfortunately, how the visceral fat rating™ is determined is not clear as it again protected by a company patent (Tanita). The correlation between chemerin levels and visceral fat rating™ is however an interesting one. It may be

that the amount of visceral fat present in an individual is the most important variable in determining a person's chemerin levels. In order to calculate visceral fat levels it would have been necessary to measure WHR which is thought to be a more accurate predictor of cardiovascular risk rather than BMI (114). Unfortunately WHR was not recorded in the cohort of patients in this study and is admittedly a shortcoming. Nonetheless chemerin levels being positively correlated with the visceral fat rating™ score is further evidence of chemerin as an adipokine. Higher visceral fat levels seem to lead to a micro-environment in the tissues that is optimal for the possible development of PCa. As can be seen in Figure 7-11 to Figure 7-13 and Table 7-3 there is a significant positive correlation between the visceral fat rating score and an individual's weight, BMI and percentage body fat (PCC = 0.437, 0.669 and 0.895 respectively. There is also a weak positive correlation between visceral fat score and lean body mass, (PCC = 0.211, Figure 7-14) as calculated by the Hume equation. As chemerin levels are significantly positively correlated with the visceral fat rating™ this is a significant finding. This effectively demonstrates that chemerin is correlated with several body parameters and thus behaves how an adipokine would be expected to behave. It is interesting that out of all the body parameters investigated during this study chemerin was only significantly positively correlated with the visceral fat rating™ and not weight, BMI, percentage body fat or lean body mass (although there was a weak positive correlation between chemerin and BMI and between chemerin and percentage body fat). Given that there is such a strong positive correlation between the visceral fat rating™ and an individual's weight, BMI and percentage body fat one might have expected a significant correlation between these parameters and chemerin. This may be due to the fact that, although there is a significant positive correlation between chemerin levels and the visceral fat rating™ score the correlation is possibly not as strong as it could be. As mentioned above chemerin levels are also positively associated with metabolic (not chronological) age and metabolic age appears to positively correlated with the visceral fat rating™ (PCC = 0.883, $p < 0.01$, data not shown). Interestingly chronological age is positively associated with the visceral fat rating™ (PCC = 0.421, $p < 0.01$, data not shown).

The significant correlations detected between visceral fat rating™ and body parameters may in some part be attributable to metabolic or chronological age and this must be borne in mind when evaluating this data. There is no correlation between chronological age and chemerin levels (data not shown) in the data presented here, however Hu et al., have shown that in 116 diabetic patients together with 38 healthy volunteers age is positively correlated with chemerin levels ($r = 0.303$, $p < 0.001$) (115). These findings may be affected by the fact that the vast numbers of patients in this study are diabetic and this may play a significant part in determining the chemerin levels as diabetic patients tend to have characteristics of the metabolic syndrome.

Conclusion

As has been demonstrated using the data presented previously I have been able to demonstrate a potential novel role for the adipokine chemerin in prostate carcinogenesis. Using well known PCa cell lines as well as benign and malignant human prostate tissue I have been able to demonstrate through basic scientific techniques the presence of chemerin, together with its receptor: ChemR23 DNA and protein in PCa cell lines and human prostate tissue. This finding is fundamental to the hypothesis that chemerin, as an adipokine, plays a role in obesity-driven prostate carcinogenesis. As mentioned in the introductory chapter obese men appear to have a poorer prognosis when diagnosed with PCa. This is perhaps due to the inherent difficulties in diagnosing PCa in obese men in a timely fashion e.g. due to difficulties in performing an adequate prostate examination, and thus obese patients present with more advanced disease. It may however, be also due to the interplay between adipose tissue and tumour cells in terms of modification and manipulation of tumour biology by adipokines.

Using basic cell assays I was able to demonstrate the positive effects that chemerin has on cell proliferation in human PCa cell lines. Chemerin appeared to significantly increase proliferation of both PC3 and LNCaP cells after 24 hours compared to basal (0nM chemerin). There were no significant effects demonstrated on cell invasion in either cell line however there was a significant increase in “wound” healing or migration in PC3 cells when treated with chemerin after 24 hours. Prevention of apoptosis is a key feature of carcinogenesis and in the LNCaP cell line there was a significant reduction in the LNCaP cell line after 4 and 12 hours treatment with chemerin. There appeared to be a reduction of apoptosis in PC3 cells as well however this failed to reach statistical significance. It is possible that these effects are mediated through up regulation of the oncogene bcl2 with increased expression demonstrated in both cell lines after stimulation with chemerin for 24 hours. There was also some evidence of downregulation of the TSG p53 but only in the LNCaP cell line. This

suggests that cellular proliferation and apoptosis is under control by multiple regulatory mechanisms but suggests a possible mechanism behind the positive findings discovered.

Investigation of common signal-transduction pathways in PCa cell lines suggested that, in the PC3 cell line at least, there may be a role for AKT and ERK1/2 activation by chemerin. These molecules play a key role in cellular processes such as growth and proliferation so the fact that there appears to be increased activation of these molecules by chemerin is potentially significant. There appeared to be no change in activation of these molecules in the LNCaP cell line nor was any activation of molecules demonstrated within the mTOR pathway in PC3 cells. Matrix Metalloproteinases (MMP) appear to play a key role in the pathogenesis of metastatic disease in PCa. There appears to be some limited evidence from the data published that chemerin may cause up regulation of MMP2 in LNCaP cells as demonstrated by WBA. The findings appeared to be less consistent in the PC3 cell line and there was even some evidence that there was reduced secretion of MMP with chemerin stimulation as demonstrated by gelatin zymography; clearly further work is required in this area.

Anterior gradient 2 (AGR2) appears to confer a metastatic phenotype in multiple adenocarcinomas, not least PCa. I was interested to investigate the effects of chemerin on AGR2 expression in PCa cells. Chemerin appeared to increase expression of AGR2 cDNA and protein at varying time points and doses of chemerin. This is a potentially interesting finding as chemerin stimulation my potentially be a reason why obese men tend to do poorly when diagnosed with PCa. AGR2 is potentially a novel biomarker in PCa diagnosis as it is secreted in the urine. AGR2 appears to be found at higher levels in the urine of men who have PCa and so may be particularly useful in the diagnosis of men who are at risk of prostate cancer in whom previous negative prostate biopsies have been performed.

From the limited clinical serum samples there was evidence, in keeping with previous published data, that chemerin is positively correlated with increasing % body fat and to a

lesser extent BMI. One would expect that, as an adipokine this would be the case and suggests that chemerin may have a role to play in obesity-related PCa. It must be appreciated that there are limited numbers in my cohort of patients and one would expect only significant changes between the two groups (benign and malignant) to be demonstrated, which hasn't been the case.

Overall I acknowledge that there are some limitations with the data that I've presented. Compared to other scientific papers regarding the cellular basis of prostate cancer the amount of human participants recruited to the study is possibly not sufficient enough to give the study sufficient power. I now appreciate the time it takes to fully counsel participants for inclusion into research and then to subsequently obtain blood and tissue samples. On the whole, patients when approached were more than willing to be involved and so further help with participant recruitment would have been invaluable. I would have preferred to perform further experiments exploring the effects of chemerin in a *time*-dependent manner as well as a dose-dependent manner. Further data could have therefore been obtained to strengthen the role that chemerin plays in the development of prostate cancer. Certain experiments (especially in regards to the western blot data) may need to be repeated in the future in order to improve the accuracy of the data and to allow stronger conclusions to be drawn.

With the above taken into consideration there is plenty of scope for further work into this project. With obesity being such a major public health concern and with rates of prostate cancer fairly stable, despite the introduction of PSA testing, the need for markers to indicate aggressive disease at an earlier stage is greater than ever. I would also be interested to explore the further outcome of the participants included in the study to see whether there is any change in behaviour of the disease in the men with prostate cancer according to their chemerin levels. I would also be interested to know whether the men without prostate cancer at inclusion into the study have an increased risk of being diagnosed with prostate cancer in subsequent months or years.

References

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Abbreviations

(c)DNA	(complementary) deoxyribonucleic acid
(m)RNA	(messenger) Ribonucleic acid
ADT	androgen deprivation therapy
AGR2	Anterior gradient 2
APC	Antigen Presenting Cells
ATP	adenosine triphosphate
BAT	Brown adipose tissue
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-2 L1	Bcl-2-like protein 1
BD	Becton Dickinson
BME	β-Mercaptoethanol
BMI	Body mass index
BMR	Basal metabolic rate
BPE	Benign prostatic enlargement
BPH	Benign prostatic hyperplasia
BPT	Benign prostatic tissue
BSA	Bovine serum albumin
CAD	Coronary artery disease
CMKLR1	Chemokine-Like Receptor 1
CRP	C-reactive protein
CT	Computed tomography
DM	Diabetes mellitus
EBP1	ErbB3 binding protein 1

ECM	Extracellular matrix
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FCS	Fetal Calf Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
HDL	High-density lipoprotein
HIV	Human Immunodeficiency Virus
HMEC	Human microvascular endothelial cells
HRP	Horseradish-peroxidase
HRPC	Hormone-refractory prostate cancer
HUVEC	Human umbilical vein endothelial cells
IFN-α	Interferon-α
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance
IHC	Immunohistochemistry
IHD	Ischaemic heart disease
IL	Interleukin
LA	Leuprorelin acetate
LBM	Lean body mass
LPA	Lysophosphatidic acid
MAP2K1/MEK1	Dual specificity mitogen-activated protein kinase kinase
MAPK	Mitogen activated protein kinases
MetS	Metabolic syndrome
MMP	Matrix metalloproteinases
mTOR	mammalian target of rapamycin

NEJM	New England journal of medicine
NGT	Normal glucose tolerance
OHS	Ovarian hyper stimulation syndrome
PAR	protease-activated receptors
PBS	Phosphate buffered Saline
PCa	Prostate cancer
PCC	Pearson correlation coefficient
PCOS	Polycystic ovarian syndrome
PI3K	Phosphatidylinositol 3-kinase
PIN	Prostatic intraepithelial neoplasia
PKB/AKT	Protein kinase B
PMA	phorbol 12-myristate 13-acetate
PSA	Prostate-specific antigen
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RARRES2	Retinoic Acid Receptor Responder protein 2
RIPA	Radio-Immunoprecipitation Assay
RRP	Radical retro-pubic prostatectomy
SC	Subcutaneous
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIV	Simian Immunodeficiency Virus
SNP	Single-nucleotide polymorphisms
T2D	Type 2 diabetes
TBS	Tris-buffered saline
TIG	Tazarotene-Induced Gene

TNF	Tumour Necrosis Factor
TRUS	Trans-rectal ultrasound
TSG	Tumour Suppressor Gene
TURP	Transurethral resection of the prostate
VEGF	Vascular Endothelial Growth Factor
VF	Visceral fat
WAT	White adipose tissue
WBA	Western Blot Analysis
WHO	World health organisation
WHR	Waist to hip ratio

University Hospitals Coventry and Warwickshire NHS Trust

THE UNIVERSITY OF WARWICK

Appendix A PARTICIPANT INFORMATION SHEET

Study title: The Role of Metabolic Related Molecules in Prostate Cancer

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

We are investigating processes that connect certain molecules with the development and progression of prostate cancer, by looking at molecules in the blood, urine and prostate tissue. This does not necessarily mean that we suspect you to have, or expect to diagnose you with prostate cancer.

Why have I been chosen?

You have been chosen because you are going to have a prostate operation or biopsy. As part of the operation, a small amount of prostate tissue will be taken for research. This is normally excess tissue (in the case of a prostate operation) not required for diagnostic purposes. Our study, which will not alter the nature or outcome of your surgery, would like to use parts of the tissue that would otherwise go to waste. If you are having a biopsy we would like to take two *extra* samples for research purposes (about the size of a matchstick). We would also like to take a small sample of your blood (equivalent to three teaspoons) and urine before and after you undergo surgery.

Do I have to take part?

It is entirely up to you to decide whether or not to take part in our study by donating blood, urine and prostate tissue. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

Before the operation or biopsy a small sample of blood will be taken as well as a urine sample. Following the procedure, the researchers will receive some of the prostate tissue. Following your procedure a repeat blood & urine sample will be taken some weeks later in outpatients. This equates to two *extra* blood tests and one *extra* urine test additional to that needed for clinical purposes.

What do I have to do?

All that is required, should you decide to take part, is to donate blood, urine & prostate tissue so that this can be used in our study. We would also be grateful if we could also measure your height, weight & lean body mass.

What are the side effects of any treatment received when taking part?

If you have surgery there are no *extra* side effects other than those expected with the procedure itself. If you have a biopsy there is a theoretical increased risk of bleeding or infection when having 2 extra biopsies (in addition to the standard 10 biopsies).

What are the possible disadvantages and risks of taking part?

Other than the risks explained to you during the normal consenting process regarding your procedure, there are disadvantages of giving up your time and having blood, urine & measurements taken.

What are the possible benefits of taking part?

There are no direct benefits to you for taking part in our study. However, it is hoped that the results of our research will benefit patients in the future, and result in a better understanding of the development of prostate cancer.

What if something goes wrong?

There is no reason to suggest that anything would go wrong by you taking part in our study. The samples obtained during your operation are normally removed as part of the operation. We wouldn't expect a problem or complication *additional* to those of the operation or biopsy that has already been explained to you. If there is a problem regarding your procedure or operation then the liability lies with the trust providing that care. If there is a problem with a research-related activity e.g. taking consent, then of course the liability lies with the research group.

Who do I contact to make a complaint?

The researchers can be contacted to discuss a problem, please see their details below. If you wish to make a formal complaint then please contact the Patient Advice and Liaison Service (PALS), University Hospitals Coventry and Warwickshire NHS Trust, Ground Floor, University Hospital, Clifford Bridge Road, Coventry, CV2 2DX, www.pals.nhs.uk

Will my taking part in this study be kept confidential?

Any information which is collected about you in connection with our study will be kept strictly confidential and will not include details of your name or address. Details required are your age, sex, height, weight and lean body mass measurements. Any information about you which leaves the hospital/surgery will have a research number only, so that you cannot be recognised in any way and the sample cannot be linked with your name.

What will happen to the results of the research study?

The results of our study will be presented in conferences and published in scientific journals, if you are interested in obtaining these results or a report on the research please contact Dr H. Randeva at the University of Warwick (details given below). The results will be anonymous, your consultant and the researchers will not be able

to identify you individually, and it will be impossible to recognise that you have taken part.

Who is organising and funding the research?

The study is organised by the department of Urology at University hospital Coventry. It will be funded by grants awarded to Mr Jefferson (UHCW) and Dr Harpal Randeva (Warwick Medical School).

Who has reviewed the study?

An approved Research Ethics Committee has reviewed the study and approved it. It has also been reviewed independently by scientific experts with knowledge of this area of research. The Medical Advisory Committee of the Warwickshire Nuffield Hospital and the Hospital Manager has also approved this study. If you require details of these reviews please contact Dr H Randeva at the University of Warwick (details given below).

Finally

We are very grateful to you for considering taking part in this study. Should you decide to take part, you will be given a copy of this information sheet as well as the signed consent for to keep.

Contacts:

University Hospitals Coventry & Warwickshire (UHCW) NHS Trust
Clifford Bridge Road
Coventry
CV2 2DX
Tel: 02476 965110
Fax: 02476 964697

The University of Warwick
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Mr KG Williams
Mr S Patel
Mr K Desai
Mr K Jefferson
Dr HS Randeva

University Hospitals Coventry and Warwickshire NHS Trust

THE UNIVERSITY OF WARWICK

Appendix B CONSENT FORM

Title of Project: The Role of Metabolic Related Molecules in Prostate Cancer

Name of Researchers:

Mr Kevin Williams / Mr Snehal Patel / Mr Ken Desai / Mr Kieran Jefferson / Dr Harpal S Randeva

Please initial:

1. I confirm that I have read and understand the participant information sheet (version 3 August 2010) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that details will be taken concerning my age, sex, height, weight and lean body mass, however my name will not be linked in any way with the sample and the information will be kept confidential. ☐

4. I understand that all samples collected from this study may be stored and used in new projects in the future. ☐

5. I understand that data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part of this research. I give permission for these individuals to have access to my records. ☐

6. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature

1 for patient; 1 to be kept in hospital notes

Appendix C Scatterplots for serum chemerin levels against multiple body parameters

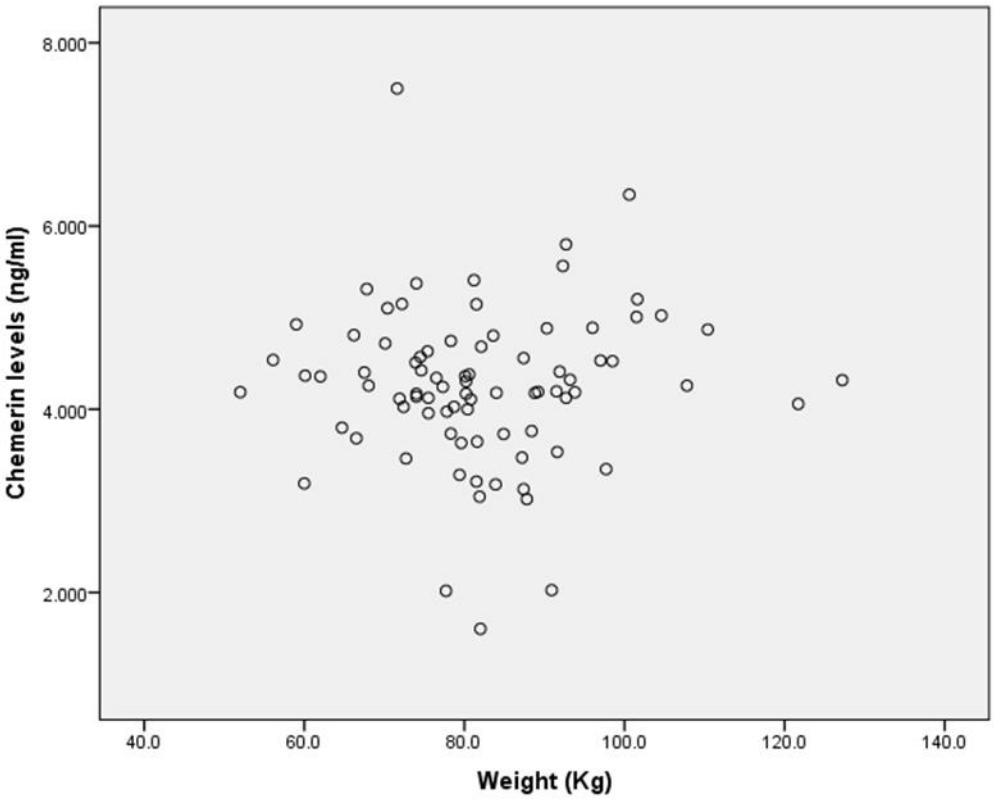

Figure C-1: Scatterplot demonstrating the relationship between serum chemerin levels (ng/ml) and weight (Kg).

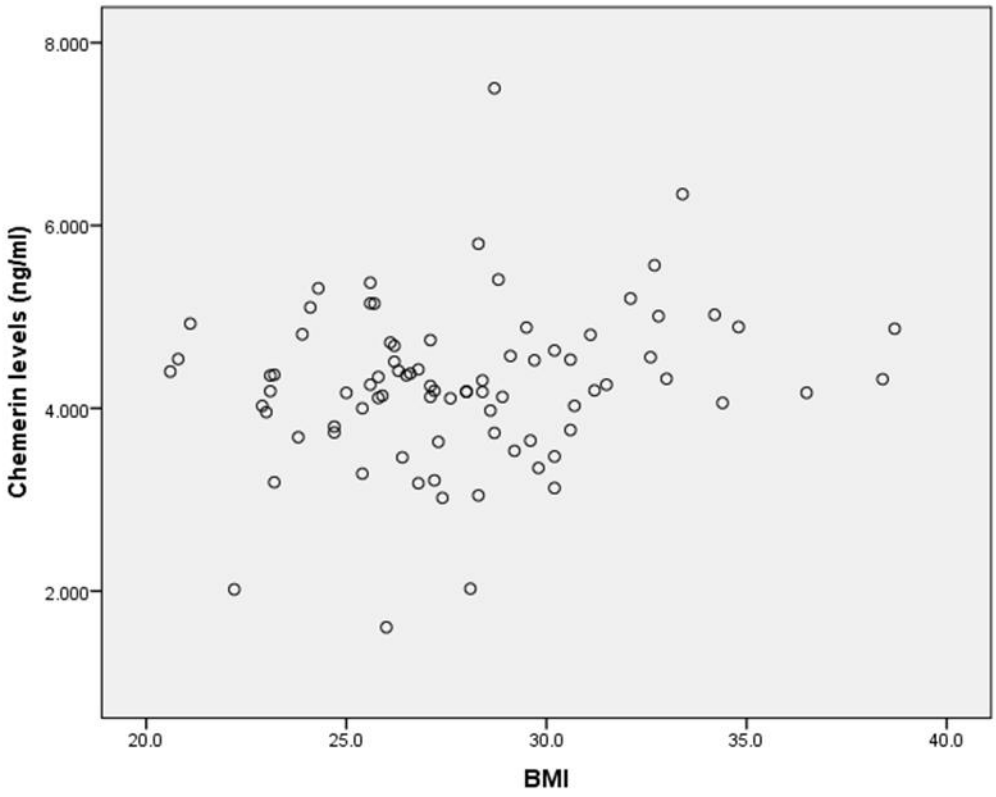

Figure C-2: Scatterplot demonstrating the relationship between serum chemerin levels (ng/ml) and BMI (Kg/m^2).

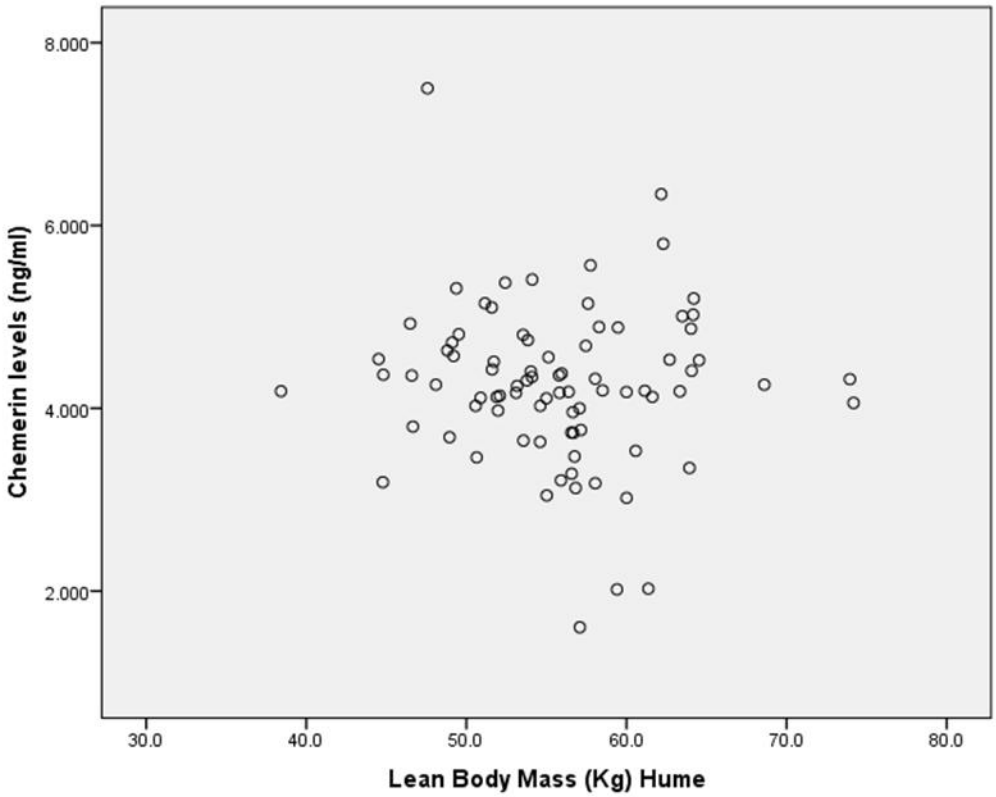

Figure C-3: Scatterplot demonstrating the relationship between chemerin levels (ng/ml) and lean body mass (Kg).

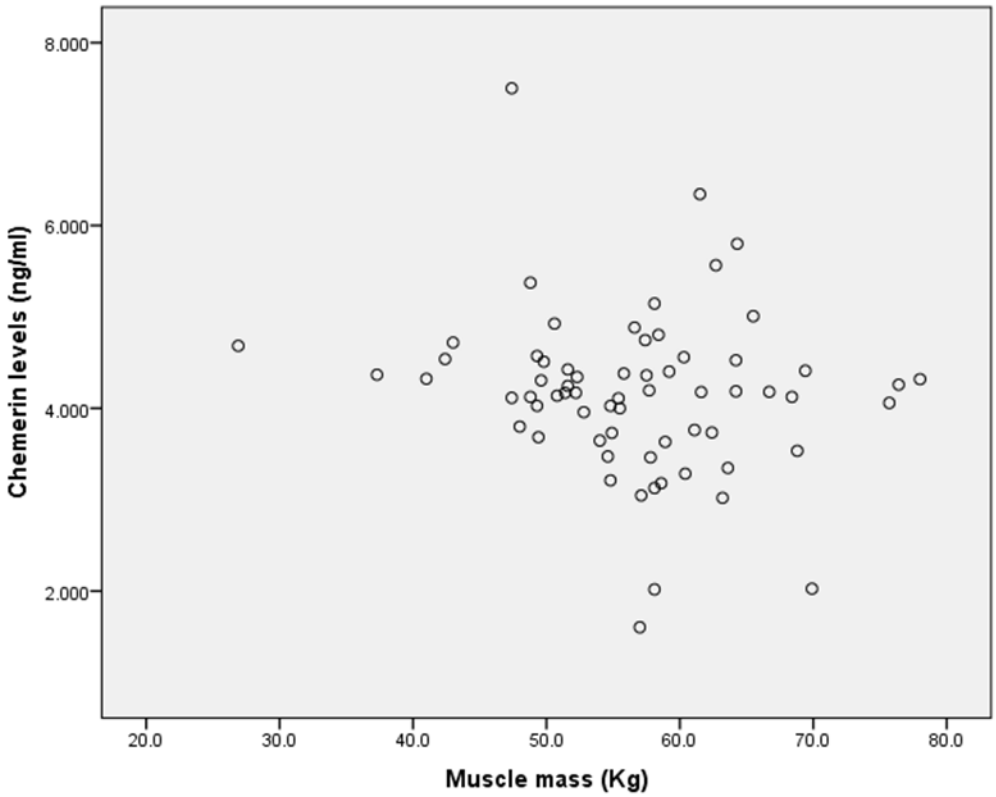

Figure C-4: Scatterplot demonstrating the relationship between chemerin levels (ng/ml) and muscle mass (Kg).

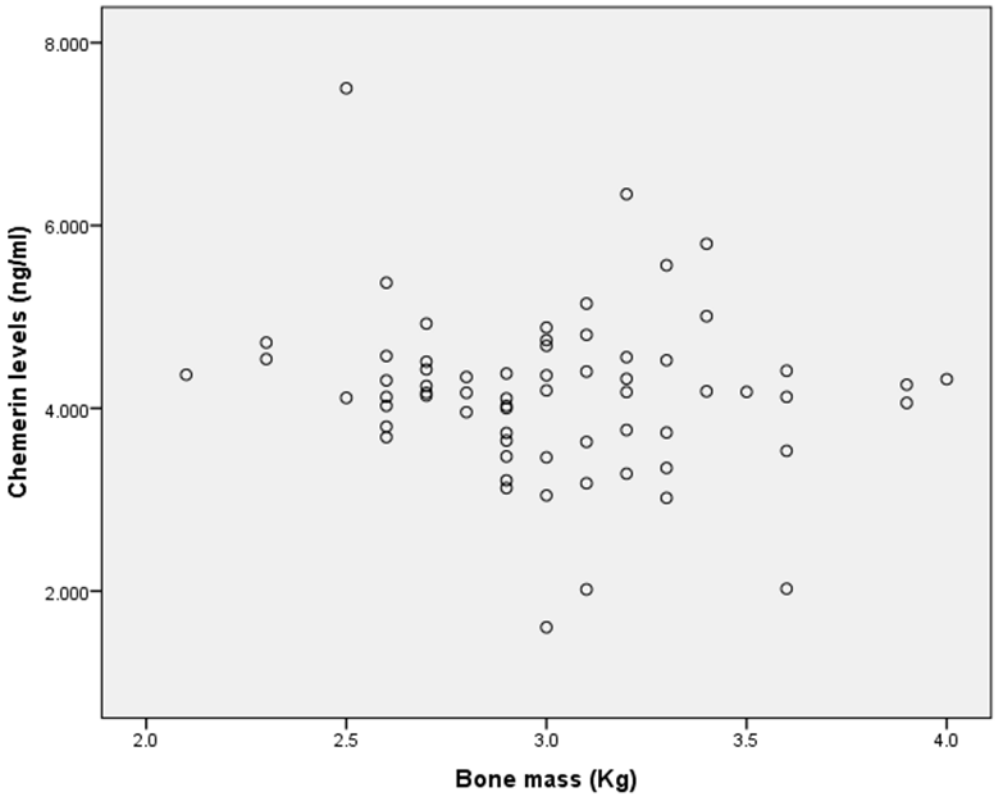

Figure C-5: Scatterplot demonstrating the relationship between chemerin levels (ng/ml) and bone mass (Kg).

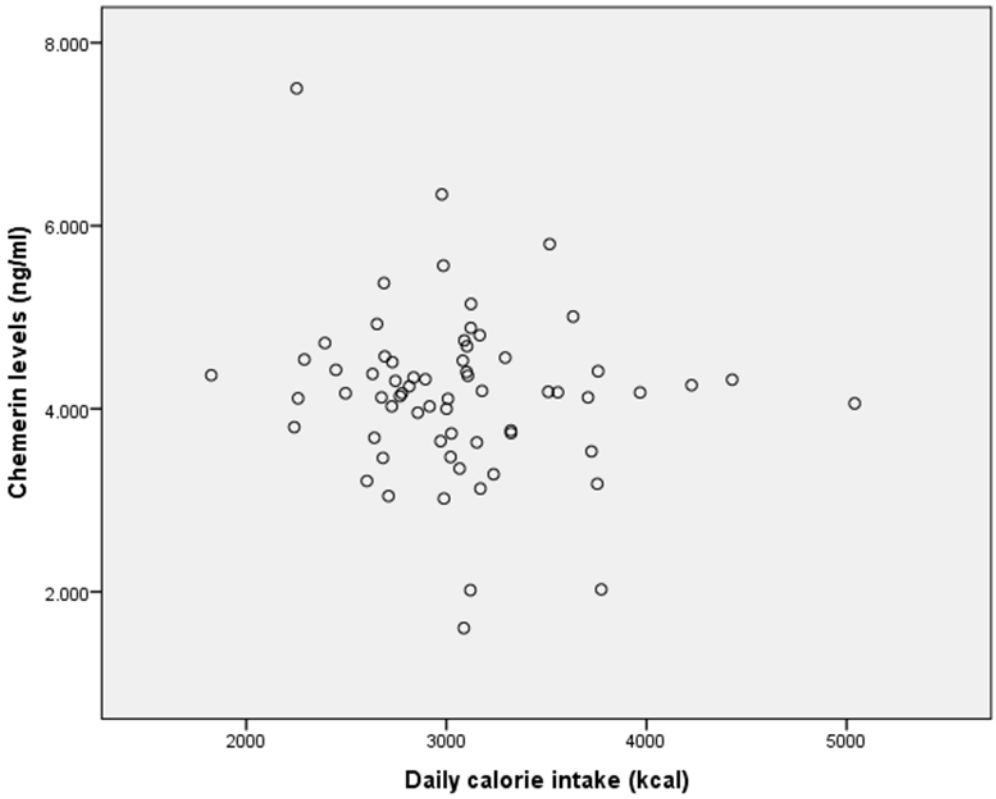

Figure C-6: Scatterplot demonstrating the relationship between chemerin levels (ng/ml) and daily calorie intake (kcal).

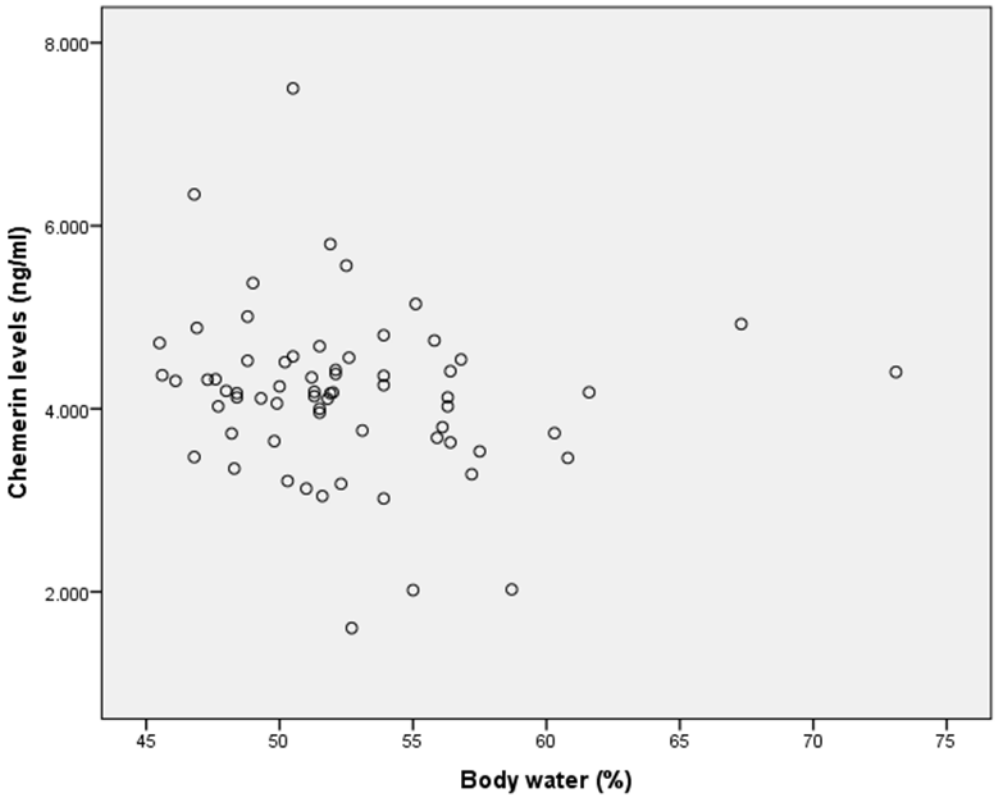

Figure C-7: Scatterplot demonstrating the relationship between chemerin levels (ng/ml) and body water (%).