DEVELOPMENT OF A CHEMOKINE-LIKE RECEPTOR 1 BIOASSAY TO STUDY THE IMPACT OF OBESITY ON ACTIVE CHEMERIN PRODUCTION IN OBESE HUMANS

by

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ABSTRACT

Prochemerin is an adipose-secreted molecule that is cleaved by extracellular proteases of the inflammatory, coagulation, and fibrinolytic cascades to active chemerin. Plasma and serum total chemerin (prochemerin + active chemerin) is increased in obese humans suggesting that chemerin may contribute to obesity-associated diseases. The effect of obesity on the production of active chemerin is unknown, given a lack of assays that specifically measure active chemerin in biological fluids. We optimized the chemokine-like receptor 1 (CMKLR1) bioassay to measure active chemerinin human plasma and serum and used this assay to determine that active chemerin is increased in obese vs. normal weight humans and in serum vs. plasma. Active chemerin chemerin concentrations were more strongly correlated with waist to hip ratio (WHR) than body mass index (BMI). This supports the potential for modified chemerin signalling and function in obese individuals with central obesity.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AUC	Area under the curve
BMI	Body mass index
CCRL2	Chemokine receptor-like 2
CMKLR1	Chemokine-like receptor-1
CRP	C-reactive protein
CVD	Cardiovascular disease
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FFA	Free fatty acids
GPR1	G-protein coupled receptor-1
PEI	Polyethylenimine
T2DM	Type 2 diabetes mellitus
TEV	Tobacco etch virus
TNF	Tumour necrosis factor
WHR	Waist to hip ratio

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CHAPTER 1: INTRODUCTION

1.1 EPIDEMIOLOGY OF OBESITY

Obesity is a condition characterized by excess white adipose tissue and low-grade systemic inflammation. With 29.8% of the world's population overweight or obese, obesity is being acknowledged as a modern day epidemic[1]. It is estimated that from 1980-2013, the number of obese or overweight adults has risen from 875 million to 2.1 billion. What is especially alarming is that during this time no country has had success in decreasing the proportion of the population that is obese[1]. In line with these global trends, the proportion of obese adult Canadians has risen from 10 to 25% over the past 20 years[2-4]. Due to the dysregulation of metabolic and cardiovascular functions that are associated with increased adiposity, obesity is an independent risk factor for other diseases including type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). As a result of obesity-associated illness, obese persons utilize more prescription medications and place a substantial burden on health care resources. For example, in Canada, it has been shown that the estimated direct economic costs of obesity account for 3% of total health care expenditures[5].These trends outline the vital need for research aimed at preventing and treating obesity and obesity associated conditions.

Once considered a disease of the affluent, obesity is now encumbering a large number of low- and middle-income families, especially in urban locations[6]. A major factor that contributes to obesity is the combination of physical inactivity and intake of calorie-dense foods. In low- and middle-income families, consuming high-fat, high-sugar, high-salt, and energy-dense foods can result in financial savings. The downside of this is that these

foods are typically lower in nutrient content and more conveniently accessed. Furthermore, due to the progressively more sedentary nature of many forms of work, varying methods of transportation, and increasing urbanization, physical activity has become less available, accessible, and affordable. Combined, these aspects of society have contributed to the current culture, which supports the development of obesity.

1.2 CLINICAL DIAGNOSIS OF OBESITY

A person's weight is reflective of their energy balance, which in the most fundamental sense is the difference between their total caloric intake and caloric output. Ingestion of food and drink comprises caloric intake, while caloric output is the sum of basal metabolism, physical activity, and adaptive thermogenesis, which refers to the body's regulated production of heat in response to changes in temperature and diet that result in metabolic inefficiency[7]. White adipose tissue increases its triglyceride (TG) stores in times of caloric excess and mobilizes these lipid stores in times of energy need. Obesity develops when there is a long-term imbalance of caloric intake exceeding caloric output, with the resulting excess energy stored in white adipose tissue as triglycerides.

The most widely used diagnostic tool for obesity is body mass index (BMI), a simple measure of weight(kg)/height(m)^2. BMI has been the time honoured measure for assessing if an individual is overweight due to the ease at which it is obtained. Individuals with BMI<18.5 are considered underweight, 18.5-24.9 normal weight, 25.0-29.9 overweight, and >30 obese. A limitation of BMI is that it assumes the variability in weight at a given height is due to changes in fat mass[8]. BMI makes no distinction

between fat and lean mass, nor does it give insight into body fat distribution. In addition to BMI, the National Institutes of Health (NIH) recommends that health care professionals assess a patients weight status based on waist circumference and risk factors for obesity-associated conditions such as blood pressure, cholesterol, blood glucose, and smoking[9]. Waist circumference should be measured near the midpoint between the lower margin of the least palpable rib and the top of the iliac crest[10]. Similar to waist circumference, waist to hip ratio (WHR) is another evaluator of obesity that can be employed alongside BMI to assess the health of an individual. WHR takes the quotient of the waist circumference divided by the hip circumference, a measure taken around the widest portion of the buttocks. This ratio specifically measures central obesity, which is the accumulation of adipose tissue in the visceral cavity. This is a clinically important measure as excess fat in this abdominal region is strongly linked to the negative health outcomes associated with obesity[11].

1.3 OBESITY TREATMENT

Obesity is a complex condition with genetic, environmental, behavioural, and biochemical influences, rendering clinical interventions relatively unsuccessful. The first option for treatment in obesity is always lifestyle changes, particularly dietary, exercise, and behavioural modifications. However, adherence is difficult and long-term benefits are modest[12]. This may cause a patient to seek pharmacotherapy in conjunction with lifestyle interventions, or bariatric surgery in extreme cases of obesity. Bariatric surgery is indicated for weight loss in morbid obesity (BMI>40), but cost barriers prevent many low-income individuals from seeking this option. In Canada, pharmacological treatment

for obesity is recommended for individuals with BMI>27 with risk factors for T2DM (impaired glucose tolerance) and CVD (high blood pressure, low high-density lipoprotein(HDL)-cholesterol) and BMI>30 as an adjunct to lifestyle modifications when they have not achieved weight loss of 0.5 kg per week 3-6 months after altering their lifestyle[13].

The obesity epidemic combined with shortcomings in weight loss and poor efficacy and toxicity of older anti-obesity agents has provided great incentive for the development of new agents. Past, present and potential anti-obesity drugs typically operate through one or more of three main mechanisms: increasing the body's metabolism, suppression of appetite, and inhibition of absorption of specific nutrients in ingested food. Past drugs no longer available in Canada or the United States include 2,4-Dinitrophenol (DNP), sibutramine, and rimonabant. DNP exerts its anti-obesity effects by increasing the body's metabolic rate through the uncoupling of mitochondrial oxidative phosphorylation[14, 15]. As a result of severe adverse effects such as hepatotoxicity, nephrotoxicity, cardiotoxicity, and agranulocytosis, DNP was banned by the food and drug administration (FDA) in 1938[15]. Sibutramine is an oral anorexiant that operates through selective serotonin and norepinephrine reuptake inhibition to suppress appetite. Sibutramine was marketed until 2010 when it was discovered that it was associated with a greater rate of adverse cardiovascular events[16]. A novel mechanism of action mediated through cannabinoid inverse agonism was brought on by the introduction of rimonabant. However, in addition to desired weight loss and an improved metabolic profile (improved glycemia, decreased blood pressure, and improved cholesterol), clinical

trials showed the use of rimonabant could trigger the unwanted effects of depression and anxiety[17]. A study of the long-term effects of rimonabant found an increased rate of serious psychiatric effects including suicide associated with the use of the drug and it was subsequently withdrawn from the market[18]. Marketed in 2013, lorcaserin causes suppression of appetite through agonism of the serotonin $5HT2_C$ receptor within the central nervous system[19, 20]. Treatment with lorcaserin resulted in modest weight loss of 3.2 kg and a BMI decrease of 1.2 kg/m^2 after 1 year compared with placebo[21]. While still new, relatively few cardiovascular side effects are predicted because of the selectivity for the $5HT2_C$ receptor[22]. Other anti-obesity drugs operate by inhibiting the ability of the body to absorb certain nutrients of ingested food (e.g. orlistat which blocks fat breakdown and subsequently prevents fat absorption)[23]. Combining a number of long term studies on the efficacy of orlistat showed a 9% reduction in body weight in orlistat-treated patients compared to 5.5% in placebo[24]. Another recently approved pharmacological agent for obesity is the extended release combination of phentermine/topiramate, which was approved by the FDA in 2012. Phentermine, which as a single agent has been approved for the short term treatment of obesity since 1959, operates through increasing norepinephrine in the hypothalamus[16]. It is thought that topiramate reduces appetite via its effect on gamma-aminobutyric acid receptors[24]. Significant weight loss was achieved after 2 years using this drug combination [25]. A pharmacological approach currently under development is a combination of bupropion and the opioid agonist naltrexone. This drug combination reduces food intake and has been shown to induce weight loss in 4 phase III clinical trials[26-29]. However, studies have shown that naltrexone/bupropion (tentatively named Contrave) increases blood

pressure and heart rate, and so an FDA-mandated investigation into potential cardiovascular complications is ongoing[24].

Despite the promise held by these new anti-obesity agents, it is still too early to make a firm conclusion regarding efficacy and toxicity in real world populations. As a result of these shortcomings in weight loss and diet management products and services, research that aims to better understand obesity as a disease by examining the endocrine function of fat tissue and to identify new therapeutic drug targets for obesity and obesity-related diseases remains vital.

1.4 ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

The two types of fat in the mammalian body are brown and white adipose tissue. Brown adipocytes are comprised of many small lipid droplets and large iron-containing mitochondria, which give their characteristic brown colour. In humans, the use of fluorodeoxyglucose positron emission tomography (FDG PET) has revealed that brown adipose depots are located mainly in the supraclavicular and neck regions, with some additional depots in the paravertebral, para-aortic, mediastinal, and suprarenal areas[30]. The primary function of brown fat is to expend heat via the uncoupling protein-1 (UCP1) in the process of non-shivering thermogenesis in newborns. Given their role in dissipating energy and recent evidence of brown adipose tissue in adult humans, new research aimed at recruiting brown adipocyte numbers is being investigated as a strategy for weight loss in obese adults [31, 32]. In contrast to brown adipocytes, white adipocytes contain a single large lipid droplet, have few mitochondria, do not express UCP1, and

their primary function is energy storage. White adipose depots are located beneath the dermis of the skin (subcutaneous adipose tissue) and around the internal organs in the abdomen (visceral adipose tissue)[33].

Our view of white adipose tissue, which makes up the majority of fat in the body[34], has changed a great deal over the past two decades. Initially thought of as being only an inert energy storage tissue, it is now recognized that white adipose tissue is an endocrine organ that secretes many biologically active proteins, collectively termed adipokines. Adipokines comprise a wide range of proteins including chemerin, leptin, resistin, tumour necrosis factor (TNF), adiponectin, and interleukin-6 (**Figure 1**)[35]. By definition, adipokines are biologically active molecules produced by adipose tissue that have autocrine or paracrine functions within adipose tissue. Adipokines also exert endocrine effects on other tissues such as the brain, liver, and skeletal muscle[36]. Adipokines regulate biological functions including: appetite and energy balance, insulin sensitivity, lipid metabolism, inflammation and blood pressure[37-39]. It is well established that the degree of adiposity has a major influence on adipokine secretion[40]. For example, the expression of TNF, one of the many inflammatory markers that regulate obesity-associated inflammation, is increased in obese adipose tissue and contributes to insulin resistance and the pathogenesis of T2DM[41]. As a result, adipokines may provide a molecular link between increased adiposity and the development of T2DM and CVD.

1.5 CHEMERIN

In 2007, the chemoattractant protein chemerin was identified as a novel adipokine[42-44]. Originally identified as an up-regulated gene in psoriatic skin, chemerin is also known as tazarotene-induced gene 2 or retinoic acid receptor responder 2. Since its discovery as an adipokine, it has been demonstrated that chemerin facilitates a diverse range of biological processes including immune cell recruitment and inflammation, angiogenesis, bone remodelling, and cell growth and differentiation[45-51]. These functions have been attributed to the signalling pathways induced by chemerin binding to and activating the G-protein-coupled receptor (GPCR) chemokine-like receptor 1 (CMKLR1).

1.5.1 CHEMERIN NOMENCLATURE

For the purpose of this thesis, the following chemerin nomenclature will be utilized from this point forward. Prochemerin will refer to the secreted inactive pro-form of chemerin composed of amino acids 21-163 of the translated human gene product. The active isoforms of chemerin will be described based on the terminal amino acid of the processed protein - e.g. chemerin$_{157}$. For the results obtained by this study, active chemerin refers to the apparent concentration of chemerin$_{157}$ equivalents as determined by the CMKLR1 bioassay. Total chemerin refers to the sum of prochemerin + active chemerin.

1.5.2 REGULATION OF CHEMERIN

In humans, chemerin is most highly expressed in the liver, lung, and white adipose tissue[42, 44]. There is intermediate expression in the ovary, and to a much lesser extent in brown adipose tissue, heart, lung, kidney, and skeletal muscle. Chemerin is synthesized as preprochemerin, a 163 amino acid protein. Following N-terminal cleavage of a 20 amino acid sequence, the 18-kDA inactive precursor prochemerin is secreted[52]. The majority of circulating chemerin is believed to exist in this relatively inactive prochemerin form[53]. Prochemerin undergoes proteolytic processing by serine proteases of the inflammatory, coagulation, and fibrinolytic cascades[54]. These enzymes are differentially expressed in a large number of diverse tissues and cell populations, and are responsible for converting the inactive prochemerin into various isoforms of active chemerin.

Chemerin activity can be defined as the ability of the chemerin product to activate CMKLR1 signalling in cell-based reporter gene or chemotaxis assays. There are multiple tools that can be used to assess chemerin activity in experimental samples. In a chemotaxis or cell migration assay, the migration of CMKLR1-expressing pre-lymphocytes towards a chemerin-containing sample of interest corresponds to chemerin activity. Alternatively, chemerin activity can be quantified through measurement of the activation of a reporter gene construct that is linked to the CMKLR1signalling pathway. In the aequorin-based calcium assay, CMKLR1-expressing Chinese hamster ovary cells that also contain mitochondrial aequorin (intracellular reporter gene) are incubated with coelenterazine, the luminescent substrate for aequorin. The cell suspension is treated with the sample of interest, and activation of CMKLR1 results in increased intracellular

calcium concentrations, activation of coelenterazine in the aequorin complex, and light emission proportional to levels of CMKLR1 activation[42, 52]. A drawback of this assay is that it is non-specifically activated by other factors in plasma and serum that trigger calcium uptake into cells. Another assay that relies on the activation of a reporter gene construct linked to CMKLR1 is the CMKLR1 "Tango" bioassay, hereafter called the CMKLR1 bioassay[55, 56]. Based on the direct mechanism of this assay it is predicted to have less non-specific activation and thus, enhanced sensitivity to detect chemerin in biological fluids compared to the aequorin assay. This assay is described in further detail in section 2.3.1.

Through cleavage at distinct sites in the C-terminus, the aforementioned serine proteases convert prochemerin into various active isoforms that vary in length and biological activity (**Figure 2**). For example, conversion of prochemerin into chemerin$_{157}$ requires the removal of 6 amino acids. This enzymatic cleavage is mediated by cathepsins K and L, human leukocyte elastase, and staphopain B. Of the identified isoforms, chemerin$_{157}$ displays the highest activity (as determined by migration assays), approximately 100-fold higher than prochemerin[52]. Chemerin$_{156}$ is slightly less active than chemerin$_{157}$, lower activity is exhibited by chemerin$_{158}$ and $_{155}$ and chemerin$_{154}$ and $_{152}$ are relatively inactive (**Figure 2**) [52, 54, 57-60]. Interestingly, prochemerin can be processed at more than one cleavage site by the same protease and some isoforms may be subject to further cleavage, meaning that chemerin can be activated in a single or sequential multi-step manner. That is, isoforms that are relatively low in activity may be processed into more active forms, and vice versa. Chemerin$_{155}$, which displays agonist activity, has been shown to be a weak antagonist in the presence of the most bioactive

isoform, chemerin$_{157}$. This suggests that the ratio of active to inactive isoforms could be a useful tool in determining chemerin bioactivity. Much of the current knowledge on chemerin processing is derived from *in vitro* studies. However, various chemerin isoforms have been isolated from biological fluids. The discovery of different compositions of chemerin isoforms in plasma (chemerin$_{155, 157, 158}$), ascites (chemerin$_{157}$), synovial fluid (chemerin$_{158}$), cerebrospinal fluid (chemerin$_{158}$) and hemofiltrate (chemerin$_{154}$) demonstrates that localized chemerin processing varies depending on the physiological context[52, 54, 58-60]. As noted by Rourke et al.[53], the majority of studies report chemerin activity as it pertains to one specific signalling pathway or function, and it remains to be seen whether the various chemerin isoforms show a discrepancy in activity on multiple pathways or functions. Further studies that specifically examine the presence of particular isoforms in disease states will help in our understanding of local chemerin activity and the biological functions of chemerin.

1.5.3 RECEPTORS AND SIGNALLING

Chemerin has been identified as a ligand for 3 GPCRs: CMKLR1, G protein-coupled receptor 1 (GPR1), and chemokine receptor-like 2 (CCRL2). In mice, CMKLR1 is expressed in immune cells such as macrophages, monocytes, dendritic cells, polymorphic mononuclear cells, natural killer cells, and T-lymphocytes where it regulates immune responses at sites of tissue injury or inflammation[50-52, 61-64]. CMKLR1 is also expressed in non-immune cells including preadipocytes, adipocytes, osteoclasts, skeletal muscle cells, vascular endothelial cells, hematopoietic stem cells, and bone-marrow derived stromal cells (BMSCs) where it facilitates a diverse range of biological functions

including cellular proliferation and differentiation, metabolism, bone-remodelling, and vascular function[42-44, 46-48, 65-67]. Similar expression patterns are found in humans. Human CMKLR1 expression is high in white adipose tissue and also shows intermediate expression in the heart, lung, placenta, lymph nodes, and spleen[42, 52]. In immune cell populations, CMKLR1 is most highly expressed in macrophage and dendritic cells. Currently, very little is known regarding the signalling pathways associated with chemerin binding toCMKLR1.Wittameret al. showed that activation of CMKLR1 by chemerin resulted in increased intracellular calcium and decreased intracellular cAMP[52]. Low doses of chemerin treatment in human endothelial cells and adipocytes stimulated the phosphorylation of extracellular signal-regulated kinase-1 and -2 (ERK1/2), which are involved in endothelial cell proliferation and adipogenesis, respectively[46, 68]. Recently, it has been shown that CMKLR1 activates signalling through both MAPK/ERK and Ras homolog gene family member A/Ras homolog associated protein kinase dependent signalling pathways (Personal communication with J Rourke). Both of these pathways are required for chemerin-mediated chemotaxis of CMKLR1-expressing L1.2 pre-B lymphocytes. It has been demonstrated that CMKLR1 deficiency in mice is linked to reduced food consumption, body weight, and adiposity[66]. Given it's many contributions to biological functions, altered CMKLR1 signalling as a result of elevated chemerin could have a number of significant physiological effects *in vivo*.

GPR1 is similar in structure to CMKLR1 and both receptors are bound and activated by chemerin with similar affinity[55]; however very little is known regarding the function and signalling pathways of this receptor. Recently it has been discovered that GPR1

contributes to the regulation of glucose homeostasis through the use of a GPR1-knockout mouse model[69]. These mice displayed exacerbated glucose intolerance compared to wild type mice despite having similar adiposity and weight. The relevance of the finding to humans remains to be determined.

A third receptor chemerin receptor CCRL2 is not believed to be a signalling receptor as it lacks an intracellular signalling domain and is not internalized following ligand binding[70]. Instead, it is thought that CCRL2 may facilitate chemerin localization by offering it to nearby cells and potentially contributing it to CMKLR1 and GPR1 mediated processes[70, 71].

Given that the vast majority of known biological functions exerted by chemerin can be attributed to CMKLR1 signalling, we have chosen to focus on the activation of this receptor as a measure of chemerin activity in our study.

1.5.4 CHEMERIN FUNCTION IN OBESITY AND DISEASE

Due to the high level of chemerin expression in white adipose tissue, we have hypothesized that white adipose tissue is a modifiable source of active chemerin in response to changes in body fat mass. This is supported by obese animal models such as ob/ob (leptin deficient) and db/db (leptin receptor deficient) mice as well as diet-induced obese mice displaying elevated serum total chemerin concentrations[65, 66, 72]; as well as human studies demonstrating significantly higher chemerin secretion from subcutaneous white adipose tissue explants from obese subjects compared to lean controls[48]. A study that quantified chemerin concentrations in women with polycystic

ovary syndrome (PCOS) showed that fat mass and not PCOS has a greater influence on serum chemerin levels[73]. Conversely, weight loss through bariatric surgery and changes in diet/physical activity that reduced adiposity resulted in a significant decrease in serum chemerin in humans[74]. Treating mouse adipocytes with TNF, an inflammatory mediator which displays increased expression in obese white adipose tissue and differentially regulates adipokines[75, 76], induced chemerin secretion while treating hepatocytes with TNF did not[72]. This suggests that despite the high expression of chemerin in the liver, the increased chemerin observed in obesity is likely to be adipose tissue derived. Additionally, white adipose tissue secretes many proteases that are able to convert prochemerin into active chemerin[77]. This suggests that not only is adipose tissue a modifiable source of chemerin, but it contains the proteolytic machinery to activate chemerin. Animal studies demonstrate that the secretory and proteolytic mechanisms of chemerin are elevated in pro-inflammatory environments[72, 78]. If a similar effect occurs in humans it could provide a potential mechanism for obese humans to produce more active chemerin.

There are a number of potential physiological effects that could be altered by the presence of elevated concentrations of active chemerin. Adipocyte hypertrophy, the enlargement of existing adipocytes, and adipocyte hyperplasia, an increase in the number of adipocytes mediate the growth of adipose tissue in obesity. One contributing mechanism to adipocyte hyperplasia is increased proliferation and differentiation of preadipocytes into adipocytes. Inhibition of chemerin or CMKLR1 signalling results in impaired differentiation of 3T3-L1preadipocytes and murine mesenchymal stem cells into adipocytes[42, 47, 79], suggesting that chemerin signalling promotes adipogenesis.

Thus it is possible that elevated chemerin in obese humans could impact adipose tissue remodelling through alteration of adipocyte differentiation.

Increased active chemerin concentrations in obesity could also contribute to adipose tissue inflammation. One of the key functions of chemerin is that it acts as a chemoattractant by recruiting immune cells such as macrophages, dendritic cells, and natural killer cells to sites of injury or inflammation[20, 30, 51-59]. Recruitment of these immune cells is characteristic of white adipose tissue inflammation observed in obesity[80-83]. In support of this idea, Ernst et al.[66]demonstrated that the loss of CMKLR1 through a ubiquitous gene knockdown affects the recruitment of various immune cells in white adipose tissue and liver. CMKLR1 deficient mice fed a low fat diet showed a decrease in dendritic cell infiltration into the liver. Liver dendritic cells influence hepatic inflammation by activating natural killer (NK) cells and T cells[84]. Both low fat- and high fat- fed CMKLR1 KO mice had significantly less infiltration of CD3+ T cells and increased infiltration of NK cells in white adipose tissue[66], indicating that this chemerin signalling pathway has physiological relevance to immune function. Furthermore, elevated chemerin is associated with many inflammatory conditions including ulcerative colitis[85], chronic pancreatitis[86], polycystic ovary syndrome[87], and liver disease[74, 88], suggesting that chemerin could play a heightened role in the pathology of these diseases in obese humans.

T2DM is a condition characterized by harmful elevations in fasting glucose concentrations and reduced insulin-stimulated glucose uptake as a result of insulin insensitivity. CMKLR1 KO mice fed a high-fat diet for 24 weeks displayed less adiposity that wild-type mice and did not show the characteristic increase in fasting glucose

typically associated with T2DM development[66]. In humans, a number of studies found circulating and adipose tissue-expressed chemerin to be significantly elevated in subjects when T2DM was also present[85, 89-91]. Additionally, reduced chemerin concentrations following surgical or diet-induced weight loss were significantly correlated with improved insulin sensitivity[90], further suggesting that chemerin plays a role in insulin sensitivity and subsequently T2DM. It is not currently known if increased chemerin in T2DM is correlative or causative in origin, as results from adipose tissue explants[87] and human skeletal muscle cell cultures[48] indicate that increased chemerin secretion could be both driven by and add to the elevated circulating insulin levels seen in T2DM[53].

Hyperlipidemia is defined by an increase in circulating TG and the LDL/HDL cholesterol ratio. Hyperlipidemia is commonly observed in obesity due to changes in lipolysis and is linked with an increased risk for CVD. Chemerin is positively correlated with circulating TG and LDL cholesterol, and negatively correlated with HDL cholesterol. However, studies examining the direct role of chemerin in CVD pathology are lacking. Some studies suggest that chemerin may provoke expression of CVD pathology markers such as intracellular adhesion molecule 1 (ICAM-1) and E-selectin, and could also play a role in the development of arterial wall defects[92-94]. Chemerin expression in both human peri-aortic and peri-coronary adipose tissue is strongly correlated with atherosclerosis[95]. Furthermore, the expression of chemerin and CMKLR1 in both vascular smooth muscle cells and foam cells of atherosclerotic plaque suggests that chemerin may play a role in the pathogenesis of atherosclerosis[96]. The elevations in circulating and localized chemerin levels seen in obesity and associated

conditions including T2DM and CVD suggest that chemerin signalling may play a pathologic role in these diseased states. However, the correlative nature of these studies offers little insight as to potential mechanisms linking chemerin to many of these conditions, signifying the need for additional research into chemerin and its signalling through CMKLR1.

1.6 RESEARCH SIGNIFICANCE

In inflammatory conditions that model conditions that are present in obesity, it was identified that mouse fat cells produce higher amounts of active chemerin leading to greater activation of CMKLR1[72]. This suggests that adipose tissue serves as a modifiable source of systemic active chemerin with changes in adiposity. This is supported by the observations of elevated total chemerin (i.e. prochemerin + active chemerin) in plasma or serum of obese rodents and humans. Given that chemerin has proinflammatory, cell migration and cell proliferation functions, elevated chemerin concentrations in obesity could contribute to aberrant CMKLR1 signalling and the pathology of obesity-associated diseases. This has not been directly demonstrated in humans, but the notion that chemerin could serve as a molecular link between obesity and metabolic and cardiovascular ailments is supported by increased plasma or serum total chemerin concentrations in humans with obesity, T2DM, hyperlipidemia and coronary artery disease[95, 97-100].

A major limitation of previous human studies examining the link between obesity and chemerin has been the reliance on single time point measures, typically a morning sample

after an overnight fast. Furthermore, these previous chemerin studies used an enzyme-linked immunosorbent assay (ELISA) capable of quantifying only total chemerin, the sum of the active and inactive forms. As such, much of the research to date has inferred a role for chemerin based on the assumption that parity exists between total chemerin concentrations and CMKLR1 activation. Given that prochemerin is relatively inactive, we argue that the quantified levels of total chemerin may not reflect the levels of chemerin that are able to bind to and activate CMKLR1. This may be especially true in situations of obesity or inflammation where prochemerin processing to active chemerin may be increased. The inference that parity exists between total chemerin concentrations and CMKLR1 activation has been brought into question by the results of two recent studies[56, 60]. For example, the study by Zhao et al. showed just a 1.58 fold increase in total chemerin concentration in synovial fluid of rheumatoid arthritis patients compared to plasma. However, by taking an additional step to measure chemerin$_{163}$, $_{158}$ and $_{157}$ using isoform-specific ELISAs a 3.33 fold decrease in prochemerin and 7.42 fold increase in active chemerin (chemerin$_{158}$ + chemerin$_{157}$) levels in synovial fluid compared to plasma was observed. The use of a standard ELISA in these samples to measure total chemerin would not have accounted for the almost 5-fold higher active to total chemerin ratio in synovial fluid compared to plasma. This result demonstrated that there is a significant shift in processing of prochemerin in the inflammatory joint disease arthritis. By this same logic, adipose tissue inflammation that occurs in obesity could have a similar effect where increased proteolytic processing could elevate circulating active chemerin concentrations. Taken together, these concepts demonstrate the need for additional research that specifically addresses how to quantify active chemerin in the

human circulation and the impact of obesity on active chemerin concentrations over time. Ultimately, this will improve our understanding of chemerin as a molecular link between obesity and associated diseases.

1.7 HYPOTHESIS AND OBJECTIVES

Given the proinflammatory conditions present in obesity, we hypothesized that obese humans have elevated plasma and serum active chemerin. In order to test this hypothesis I developed the following 2 specific research objectives: 1) to develop and optimize the CMKLR1 bioassay to quantify active chemerin concentrations in human plasma and serum, and 2) to determine the effect of obesity on the plasma and serum concentration of active chemerin in humans. The primary analysis measured active chemerin in plasma, as this reflects the chemerin concentration in the circulation. Since coagulation cascade enzymes process prochemerin and obesity is associated with a procoagulant state, obese people may be prone to form more active chemerin in situations where coagulation is activated[54, 101]. Thus, it was physiologically relevant for us to also measure serum active chemerin. Previous human studies have measured total chemerin in a single serum or plasma sample in the morning following an overnight fast. Similarly, our clinical study included morning fasting plasma and serum samples to allow comparison of measured active chemerin to published baseline values of total chemerin. The finding in mice that serum total chemerin oscillates with time of day[72]provided rationale for us to include multiple sampling time points in the experimental protocol. Given that insulin stimulates prochemerin secretion by adipocytes, we were interested whether we could detect any post-prandial increases in chemerin[87]. Overall, the clinical sampling procedures were

designed to be the first to examine if plasma and serum active chemerin increased the in the post-prandial period following breakfast, and if the changes differed between obese vs. normal weight humans.

CHAPTER 2: METHODS

2.1 SUBJECTS

The study protocol and consent form was approved by the IWK Research Ethics Board. The study called for males or females ages 18 to 55 years with a body mass index (BMI) of 20-25 (normal weight, n=4) or BMI > 30 (obese, n=4) that provided written informed consent. The study excluded subjects taking medications that enhance chemerin formation (insulin and metformin)[87] or block chemerin degradation (ACE inhibitors)[77]. Subjects with renal impairment (creatinine clearance<60 $mLmin^{-1}$), T2DM, or previous gastric bypass surgery were also excluded as these conditions may also influence chemerin concentrations independent of obesity[102].

2.2 CLINICAL PROCEDURES

Participants reported on the study day to the Clinical Research Unit at 6:00 AM following an overnight fast of 10-12 hours, as changes in nutritional status may impact circulating adipokine concentrations[66, 72, 103, 104]. On arrival, subjects had a saline lock placed and blood samples were collected at each of 07:00, 08:00, 8:30, 09:00, 9:30, 10:00, 11:00, 12:00 and 13:00. For each time point, one blood sample (4 mL) was collected into a sodium citrate tube for plasma preparation and another 4 mL blood sample into a serum tube for serum preparation. Blood samples for serum preparation were left at room temperature for 2 hours after collection to allow for clotting to occur. Blood samples were centrifuged at 2500×g for 5 minutes at 4°C to yield approximately 2

mL of plasma or serum, which were then aliquoted and stored at -70°C prior to analysis. Each subject remained in the Research Unit for the study duration and ate varying amounts of a wide selection of breakfast items at 08:00. Subjects were also assessed for anthropometric measures of body fat including BMI, waist to hip ratio and waist circumference as well as systolic and diastolic blood pressure and heart rate. Baseline TG, free fatty acids (FFA), cholesterol, insulin, C-reactive protein (CRP), and plasma glucose were quantified in each serum sample using standard methods in the Hospital Clinical Chemistry Laboratory. Concentrations of these factors in all remaining samples were quantified in our laboratory.

2.3 CMKLR1 BIOASSAY

2.3.1 PROTOCOL

This assay requires a transfection of a bacterial plasmid expressing CMKLR1 fused to a transcriptional transactivator (tTa) via a tobacco etch virus (TEV) protease recognition sequence. The DNA vector was transfected into HTLA cells, which are a HEK293-derived cell line containing a stably integrated fusion protein comprised of a TEV protease linked to human β-arrestin2, and a tTA-dependent luciferase reporter gene. Upon binding of chemerin to the receptor, there is a recruitment of the protease-tagged β-arrestin2 to the C-terminus of the CMKLR1-tTA fusion protein, resulting in tTa cleavage (**Figure 3**). This allows the tTa to migrate to the nucleus of the cell where it transcribes the luciferase reporter gene. In addition to CMKLR1, a β-galactosidase containing plasmid was co-transfected into the cells to normalize for transfection efficiency.

The initial CMKLR1 bioassay protocol that was adopted was designed for use with mouse serum and adipocyte media[56]. For the assay, 20000 HTLA cells were seeded (per well) in complete DMEM media in a 96 well plate and incubated for 24 hours at 37 º C in a humidified atmosphere of 95% air and 5% CO_2 (standard incubation conditions). The cells were then transfected by aspirating the media out of each well and treating the adhered cells in each well with a 50 μL solution comprised of (in order of addition to the transfection mixture) 10 μL serum-free optimem media, 25 ng of the CMKLR1-TL-tTA plasmid, 25 ng of a pCMV-β-galactosidase reference plasmid, 50ng of the carrier plasmid PBSK,0.1 μL of a 1 mg mL^{-1} polyethylenimine (PEI) solution and 40 μLDMEM. PEI is a cationic molecule that complexes with plasmid DNA facilitating electrostatic interactions with anionic cell surface residues and uptake of plasmid DNA into the cell via endocytosis[105]. Transfection proceeded for 24 hours under standard conditions. Under conditions optimized for the use of the CMKLR1 bioassay with human plasma and serum a batch transfection mixture containing (per well, in order of addition) 40 μL optimem, 200 ng of the CMKLR1 plasmid, 200 ng of the β-galactosidase plasmid, 20000 HTLA cells in 160 μL DMEM, and 0.4 μL PEI was used. After 24 hours, the transfection media was aspirated and replaced with 50 μL of either the plasma and serum samples diluted in optimem media 1:20 and 1:10, respectively, or 0, 0.1, 0.3, 0.6, 1, 3, 6, 10, and 30 nM recombinant human $chemerin_{157}$ purchased from R&D systems (Minneapolis, MN).For assays meant to quantify active chemerin in plasma, the chemerin standards were diluted in a mixture containing bovine plasma and 3.2% sodium citrate diluted 1:20 in optimem. For serum, the chemerin standards were diluted in a mixture containing fetal bovine serum(FBS) diluted 1:10 in optimem. The duration of the incubation for the cell

treatment step was 24 hours under standard conditions and 16 hours under optimized conditions. The treatment media was aspirated; the cells were incubated for 5 minutes with shaking (1000 revolutions per minute) in 100 µL of reporter lysis buffer (RLT) (Promega, Nepean, ON) followed by a rapid freeze/thaw cycle of 30 minutes to lyse the cells. For the luciferase assay, 10 µL of each sample lysate and RLT blank was transferred to a 96-well white luminometer plate. Eighty µL of luciferase assay reagent (Promega, Nepean, ON, CND) was auto-injected into each well and the luminescence was monitored for 10 seconds. For the β-galactosidase assay, 30 µL of each sample lysate and blank was transferred to a clear 96-well plate and incubated with 30 µL of 2xβ-galactosidase assay buffer for 15 min at 37 ºC. This assay buffer is comprised of (per 100 mL) 20 mL of1 M$NaPO_4$ pH 7.3, 100 µL 2 M $MgCl_2$, 700µL of 14.3 M 2-Mercaptoethanol, 133 mg *ortho*-Nitrophenyl-β-galactoside (ONPG), and 79.2 mL H_2O.The reaction was stopped by addition of 100 µL of 1 M $Na_2CO_3$and the absorbance at 420 nm was measured. The luciferase and β-galactosidase measurements were corrected for the respective blanks. Activation of the receptor by each standard and sample was expressed as the ratio of luciferase/β-galactosidase activity.

Using GraphPad Prism (Graph Pad Software Inc., La Jolla, CA), the active chemerin concentration of each standard was plotted against corresponding luciferase/β-galactosidase activity. The standards were fit to a 4-phase logarithmic nonlinear regression curve (log (agonist) vs. normalized response), and the concentration of active chemerin in each sample was interpolated based on its luciferase/β-galactosidase activity.

2.3.2 ASSAY OPTIMIZATION

A point of emphasis was to develop this assay for use with human plasma and serum. This involved a number of optimization steps. Optimization step #1 compared transfection of human CMKLR1 to mouse CMKLR1 to determine if there was a difference in sensitivity between the receptors. Optimization step #2 examined methods to improve cell adherence. This included the use of poly-L-lysine (Sigma-Aldrich, Oakville, ON) vs. no poly-L-lysine. Ninety-six well plates were coated with poly-L-lysine according to the protocol provided by the manufacturer .Additionally, the use of a 100 μL pipette to remove liquid out of the wells was compared to a vacuum aspirator. Optimization step #3 contrasted standard transfection with batch transfection methods. Optimization step #4 studied differences in assay sensitivity and variation following 6, 16, and 24 hour treatment times. The final optimization step, #5, aimed to find a suitable dilution factor for the human plasma and serum samples as well as a diluent for the chemerin standards.

2.4 HUMAN CHEMERIN ELISA

To determine the concentration of total chemerin in each sample, a DuoSet ELISA was employed. The assay was conducted according to the protocol provided by the manufacturer (R&D Systems). To perform the assay, 100 μL of 4.0 μg mL^{-1} mouse anti-human chemerin capture antibody was added to each well of a 96-well microplate. The plate was sealed and placed on an orbital shaker at 400 revolutions per minute at room temperature for 16 hours to allow binding of the capture antibody to the well surface.

Each well was then aspirated and washed with 300 μL wash buffer a total of 3 times. Unless otherwise indicated, incubation and washing conditions were the same for each step. Plates were blocked by adding 300 μL of reagent diluent (1% bovine serum albumin in phosphate buffered saline). The standards were prepared during this time by serially diluting a 160 ng mL^{-1} stock $chemerin_{157}$solution in reagent diluent to generate a7-point standard curve with chemerin concentrations of 0, 62.5, 125, 250, 500, 1000, and 2000 pg mL^{-1}. After a1-hour incubation in reagent diluent, 100μL of each standard and diluted sample (1:200) in duplicate was added to the plate, and incubated for 2hours.Next, 100μL of the 200 ng mL^{-1} biotinylated goat anti-human chemerin detection antibody was added. After incubation for 2 hours, 100 μL of streptavidin conjugated to horseradish peroxidase was added to each well, and the plate was covered in tinfoil to protect the light-sensitive reagent. After 20 minutes, 100 μL of substrate solution containing a 1:1 mixture of hydrogen peroxide and tetramethylbenzidine was added to each well. The plate was again wrapped in tinfoil and incubated for a further 20 minutes, this time without shaking. Lastly, 50 μL of a 2 M sulphuric acid stop solution was added to each well. The plate absorbance was immediately read at 450 nm, to detect the coloured reagent, and 540 nm, to allow for volume correction. The specific absorbance of each sample was calculated using the equation below:

$$Absorbance_{Sample} = (Absorbance_{450nm} - Absorbance_{540nm}) - Absorbance_{Blank} \quad \text{(eq. 2)}$$

Using GraphPad prism software, a 4-phase logarithmic standard curve was produced and the total chemerin concentration of each sample was interpolated.

2.5 STATISTICAL ANALYSIS

Nonparametric t-tests were used to compare standard curves in the assay optimization phase. All data are expressed as mean ±SEM. For our primary analysis, a two-way analysis of variance (ANOVA)with Bonferroni post-test was used to assess post-feeding changes in plasma and serum active and total chemerin concentration and the active/total chemerin ratios between the obese (BMI > 30, n =4) and lean (BMI < 25, n = 4) groups. For our secondary analysis, the two-way ANOVAs were repeated following the reclassification of study subjects as having central obesity (n=3) or not (n=5) based on a WHR above or below the cut off value of 0.85[10]. We performed a Spearman correlation analysis of plasma and serum active and total chemerin levels at each time point with measures of obesity including BMI and WHR and a number of metabolic parameters including cholesterol, TG,FFA, glucose, insulin, and CRP. The correlations were compared statistically to determine if active chemerin was more strongly connected to these parameters than total chemerin.

CHAPTER 3: RESULTS

OBJECTIVE 1. TO OPTIMIZE THE CMKLR1 BIOASSAY FOR USE WITH HUMAN PLASMA AND SERUM

The CMKLR1 bioassay was originally a 4 day procedure designed for use with mouse adipocyte media[72]. It was key to perform a series of optimization experiments aimed at finding a suitable vehicle and dilution factor to adjust for the testing of human plasma and serum, as well as shortening the duration of the assay while maintaining precise sample interpolation in the form of low inter- and intra-assay variability. Prior to the optimization period, negative control experiments were performed in HTLA cells that were not transfected with CMKLR1. When treated with recombinant chemerin$_{157}$ concentrations up to 30 nM, the upper limit used for the standard curves, there was no detectable luciferase/β-galactosidase activity above background (data not shown). Once samples were obtained from the pilot clinical study, further negative control experiments were conducted. HTLA cells not transfected with CMKLR1 were treated with human plasma (1:20 dilution) and serum (1:10 dilution). This resulted in no detectable luciferase/β-galactosidase activity above background (data not shown). During the optimization period, assays were performed in a sequential fashion so that results of the each optimization step could be analyzed before being incorporated into the next set of optimization experiments. HTLA cells greater than passage 25 were not used due to reduced viability.

OPTIMIZATION STEP #1: MOUSE CMKLR1 VS. HUMAN CMKLR1

The initial optimization step examined if the recombinant human chemerin$_{157}$used to generate the assay standard curves differentially activated the mouse CMKLR1 compared to its human analog. The maximum fold change in activity relative to baseline was similar if mouse (72.0±28.4) or human (61.1 ± 35.9) CMKLR1 was used, P=0.560 (**Figure 4**). The potency for chemerin$_{157}$ activation of mouse CMKLR1 (EC_{50}=1.24 ± 0.64 nM) was also statistically similar human CMKLR1 (2.87 ±0.57nM, P=0.3114). Given a trend for higher sensitivity at lower chemerin concentrations, mouse CMKLR1was used for the initial optimization experiments. Once the human samples were obtained to begin testing for appropriate dilution factor, we reverted back to using human CMKLR1, under the same optimized reaction conditions.

OPTIMIZATION STEP #2: IMPROVING CELL ADHERENCE

One initial challenge with the assay was the loss of viable cells from the plate surface as a result of the multiple manipulations required. To maximize the retention of viable cells, we explored the impact of poly-L-lysine coating of the plates. Poly-L-lysine is a cationic molecule that aids in cell adherence due to electrostatic interactions with the negatively charged cell membrane potential. **Figure 5A** shows standard curves produced with and without poly-L-lysine. There were not enough observations for statistical analysis, but after 2 experiments it was evident through microscopic evaluation that poly-L-lysine was inhibiting cell retention which was subsequently reducing the maximum assay response. As such, all subsequent assays were done with wells left uncoated.

Aspirating technique was also investigated in conjunction with coating the plates as a means of preserving viable cells. We examined the impact of removing the liquid out of the wells at each step using a vacuum powered aspirator vs. gentle manual aspiration with a (100 μL) pipette (**Figure 5B**). We found that the maximum signal response was similar regardless of aspiration method. Fluid removal for all ensuing assays was done using the vacuum aspirator due to the convenience and time efficiency it provided.

OPTIMIZATION STEP #3: BATCH VS. STANDARD TRANSFECTION

An additional mechanism for optimizing the assay was to reduce the total assay duration by combining the seeding and transfection steps in what is termed a batch transfection. Standard curves produced by batch transfection and standard transfection are displayed in **Figure 6**. The maximum fold changes produced were 254 ± 203 using batch transfection and 128± 78 using standard transfection and were statistically similar, P=0.879. Since the batch transfection did not impair assay performance compared to the standard transfection method its use was justified for all subsequent assays as it reduced assay time and tended to provide a larger dynamic assay range.

OPTIMIZATION STEP #4: TREATMENT DURATION

Modifying the time the cells were treated with the chemerin standards and chemerin-containing samples was explored as a means to further reduce the duration of the assay protocol. **Figure 7** summarizes the results obtained by studying 3 different treatment times: 6, 16, and 24 hours. The 6-hour treatment produced standard curves that saturated

at lower chemerin concentrations(approximately 1000 pM) compared to the 16- and 24-hour incubations, which had dynamic ranges up to approximately 10,000 pM. Furthermore, a lower maximal response was observed for the 6-hour versus 16- and 24-hour incubation ($P<0.05$ for both). Given the smaller range of linearity and lower maximal response, the 6-hour incubation was deemed unreliable for interpolation of sample concentrations. Due to the comparable broad range of linearity in the 16 and 24-hour treatments and similar maximum responses, we selected the 16-hour treatment duration (based on convenience) for all subsequent assays.

OPTIMIZATION STEP #5: VEHICLE AND DILUTION FACTOR

To account for nonspecific effects of the plasma and serum samples on the assay and to generate an accurate prediction of active chemerin concentration in these samples it was vital to choose a diluent for the chemerin standards that closely matched the sample compositions. Bovine plasma with 3.2% sodium citrate diluted 1:10 in optimem media was chosen as the diluent for the plasma chemerin standard curve to match the composition of citrated human plasma collected in our study. For the serum chemerin standard curve, FBS diluted 1:10 in optimem media was chosen to replicate the composition of serum samples collected in the study. The 1:10 dilution in optimem for the chemerin standards was chosen at this juncture as this dilution factor was also viewed as a potential candidate for the plasma and serum samples based on previously published work[72]. The maximum fold changes for the citrated bovine plasma (**Figure 8A**) and FBS (**Figure 8B**) standards were 245 ± 223 and 310 ± 375. These maximum values were comparable to those produced in the optimem and citrated optimem. However, the

$EC_{50}$was considerably lower when the assay was performed in the presence of FBS vs. optimem (3.99 ± 0.88 nM vs. >30 nM) or in citrated bovine plasma vs. citrated optimem (2.12± 0.70 nM vs. >30nM). This qualitative difference highlights the importance of including an appropriate diluent for the preparation of the chemerin standard curves and ultimately the determination of active chemerin concentrations in the human plasma and serum samples.

Once it was established that the FBS and citrated bovine plasma vehicles could be used, we tested control human plasma and serum samples in their corresponding vehicles in order to determine their optimal dilution range for the assay (**Figures 9&10**). Plasma and serum samples were diluted 1:5, 10, 15, and 20. For plasma, the interpolated fold-change of the 1:20 dilution consistently fell in the mid ranges of the linear regions of the standard curves. Furthermore, the coefficient of variation between biological replicates (n=3) within the same assay (intra-assay) was 5.2% and the coefficient of variation between interpolated concentrations from different assays (inter-assay, n=3) was 12.0%. These values fell within the generally accepted ranges of <10% intra-assay coefficient of variation and <15% inter-assay coefficient of variation. In serum, the 1:10 dilution typically fell within the mid ranges of the linear phases of the standard curves (**Figure 10**) with an intra-assay coefficient of variation (n=3) of 7.7% and inter-assay coefficient of variation of 14.3%.

OBJECTIVE 2: TO DETERMINE IF ACTIVE CHEMERIN IS ELEVATED IN OBESITY

CHARACTERISTICS OF STUDY SUBJECTS

Eight subjects that met the study inclusion criteria were enrolled through an initial screening visit. No subjects were excluded according to our predetermined exclusion criteria. Subjects reported to the Clinical Research Unit at the IWK and had blood collected from them on schedule as described in the methods section. Individual subject characteristics are summarised in **Table 1**. All subjects in our study were female and 4 were categorized as obese and 4 as normal weight based on BMI. Weight and BMI were significantly higher in the obese versus normal weight group. There were no differences in height, systolic and diastolic blood pressure, and heart rate. A complete blood count and assessment of metabolic profile was obtained from the 7:00 AM overnight fasting blood sample. The overnight fasting concentrations of TG, FFA, cholesterol, insulin, CRP and plasma glucose were similar in the normal weight and obese groups(**Table 2**). Serum creatinine (a biomarker of renal function) was also similar between groups. White and red blood cell counts and hemoglobin and hematocrit were similar between groups, however the mean corpuscular volume and corpuscular hemoglobin were significantly lower in the obese group (**Table 3**).Neutrophil, lymphocyte, and eosinophil counts as % of total white blood cells and platelet count and volume were comparable between groups, while red cell distribution width was significantly higher and monocyte % count significantly lower in the obese group (**Table 4**). Basophil % count, and absolute lymphocyte, eosinophil, and basophil counts were similar between groups, however the absolute neutrophil and monocyte counts were significantly lower in the obese group

(**Table 5**). The normal reference ranges for each measured parameter are shown in the tables. In the majority of cases the values fell within or near the typical reference range. CRP levels in obese subjects 5 and 8 were approximately 2- and 3-fold higher than the upper limit of the reference range and in normal weight subject 4 CRP was 5-fold higher. Another notable instance where the quantified value was well outside the normal range was the substantially elevated insulin in subject 8 (1.5-fold higher than the upper limit of the reference range). The breakfast that the subjects ate varied with respect to food items consumed, but there were no apparent differences in total caloric intake between the normal weight and obese group.

QUANTIFYING ACTIVE CHEMERIN IN HUMAN PLASMA AND SERUM

The optimized CMKLR1 bioassay was utilized to quantify active chemerin concentrations in the patient plasma and serums samples. Each sample was assayed in triplicate. The overnight fasting active chemerin concentrations for the normal weight and obese groups were 4.55 ± 0.92 nM and 6.67 ± 3.34 nM in plasma (P=0.2) and 6.49 ± 0.33 nM and 8.24 ± 1.18 in serum (P=0.005), respectively. There appears to be an initial increase in active chemerin following feeding in the obese group, and the opposite trend is seen in the normal weight subjects(**Figure 11**). Serum active chemerin appeared more stable over time and had less overall variability compared to plasma. However, given the small sample size there was not enough statistical power to determine if there was a statistical difference between pre- and post-feeding active chemerin concentrations. To increase statistical power for a between groups comparison, the active chemerin concentrations were averaged over all time points for each subject in plasma and serum

(**Figure 12A**). The average active chemerin concentration was higher in plasma and serum from obese subjects compared to normal weight (6.28 ± 0.56 nM vs. 3.93 ± 1.08 nM in plasma, P<0.001, and 8.50 ± 0.48 nM vs. 6.47 ± 1.29 nM in serum, P<0.001). Additionally, serum active chemerin was significantly higher than plasma active chemerin for both normal weight (P<0.0001) and obese (P=0.0001) groups. In addition to the average chemerin concentration, we compared the area under the curve (AUC) of the active chemerin vs. time graph for each subject to provide a measure of the overall active chemerin exposure over time. The $AUC_{0\text{-}6h}$was significantly higher in obese subjects compared to normal weight, and also in serum compared to plasma (**Figure 12B**).

To further examine the association between active chemerin levels and obesity, we correlated the active chemerin concentrations of each subject averaged over all time points in plasma and serum with BMI (**Figure 13**). The respective correlation coefficients in plasma and serum were r=0.586 and 0.693 (P=0.04and 0.02). We performed another correlation of active chemerin against WHR, an anthropometric measure of central obesity (**Figure 14**). Both plasma and serum active chemerin showed stronger correlations with WHR compared to BMI (r=0.829, P=0.02 for plasma and r=0.896, P=0.01 for serum).Consistent with the correlations, the plasma and serum active chemerin was higher (P<0.05 for both)in the obese subjects when characterized according to WHR>0.85[10](**Figure 15**).

TOTAL CHEMERIN AND ACTIVE/TOTAL CHEMERIN RATIOS

Total chemerin concentrations were measured in all plasma and serum samples using an ELISA and were assayed in duplicate. Following the overnight fast, the baseline concentrations of total chemerin for the normal weight group and obese group were 10.3 ± 1.18 nM and 14.0 ± 3.63 nM in plasma (P=0.0234)and 10.4 ± 1.05 nM and 17.0 ± 3.06 nM in serum (P<0.0001), respectively. The concentration-time profiles for plasma and serum total chemerin appeared similar to active chemerin, and were rather consistent in the fasting versus post-prandial periods (**Figure 16A&B**).A strong association between active and total chemerin concentration-time profiles is reflected by the statistically significant correlations between the measured active and total chemerin sample concentrations (r=0.694 and 0.722 in plasma and serum, P<0.05 for both). The average total chemerin concentration over all time points was higher in obese subjects relative to normal weight in plasma (14.45 ± 2.79 nM vs. 9.41 ± 0.21 nM, P=0.02) and serum (17.91 ± 1.80 nM vs. 10.90 ± 0.46 nM, P<0.0001). Furthermore, serum total chemerin was significantly higher than plasma total chemerin in the normal weight (P=0.0002) and obese (P=0.00018) groups. Similarly, the total chemerin $AUC_{0\text{-}6h}$was significantly higher in obese compared to normal weight subjects, and also in serum compared to plasma (**Figure 16C**). Total chemerin concentrations that exceed active chemerin in plasma and serum are reflected by active/total chemerin ratios that were less than unity (**Figure 17**). When grouped by BMI, similar plasma active/total chemerin ratios were observed in the normal weight and obese groups. In the normal weight and obese groups the serum active/total chemerin ratio was significantly elevated compared to plasma active/total chemerin ratio. Within serum, the active/total chemerin ratio for the normal weight group

was larger than for the obese subjects.

CORRELATIONS BETWEEN CHEMERIN AND OTHER FACTORS

Overnight-fasting plasma total chemerin was previously shown to correlate with CRP, glucose, insulin, TG, cholesterol, and FFA in humans[90, 91, 95, 97-99, 106-109].Given the small sample size of our study a correlation analysis between each measured metabolic parameter(**Figure 18**) and plasma and serum active and total chemerin concentrations over all time points was performed. The concentration-time profiles of these factors appeared different between groups so the correlation analysis was done separately for each group. In the normal weight subjects, there was a significantly negative correlation between insulin and active chemerin. In the obese subjects, active chemerin was negatively correlated with glucose and TG, and total chemerin was positively correlated with insulin and CRP (**Table 6**).

CHAPTER 4: DISCUSSION

4.1 QUANTIFYING ACTIVE CHEMERIN USING THE CMKLR1 BIOASSAY

Chemerin is a relatively new adipokine with demonstrated and putative functions in cell differentiation, immunology, and metabolism. Given these functions of chemerin and reports of higher chemerin concentrations in obese humans and animals it has been postulated that chemerin is physiologically relevant in obesity and obesity-associated comorbid conditions[44, 91, 95, 97, 98, 106]. However, the exact functions of chemerin/CMKLR1 signalling and if they are beneficial or pathogenic in obesity remain unknown. A limitation in the vast majority of human chemerin studies is the reliance on measures of total chemerin. We currently do not know if total chemerin concentrations reflect active chemerin in healthy individuals and the extent of changes in active chemerin in obesity. This is an important limitation given that active chemerin is the form that binds to and signals through CMKLR1. To address this gap in knowledge, we set out to develop a method of measuring active chemerin in human samples based on the premise that measures of active chemerin will be more useful for predicting how chemerin/CMKLR1 signalling and function changes in obesity.

Development of a new method was required because of a lack of commercially available methods to quantify active chemerin concentrations. The isoform-specific assays used to measure chemerin$_{163}$, $_{158}$, and $_{157}$ by Zhao et. al would have been useful but are proprietary and were not available for my studies through collaboration. The calcium-based aequorin assay is also not commercially available and would not have been

compatible in our study because of expected non-specific increases in intracellular calcium by plasma or serum.

The CMKLR1 bioassay was freely available to us and we have previously used it successfully for mouse serum and mouse cell culture[72].Going through the optimization steps was vital in assuring that the assay was accurately quantifying active chemerin in human plasma and serum. The use of untransfected cells as negative controls were important validation experiments that showed the result I was measuring was due to activation of the receptor and not a non-specific alteration in the reporter-gene system. The difficulties initially experienced in obtaining high signal output meant that it was key to explore methods to improve assay sensitivity. Given that the assay is cell-based, it was vital that cell viability be maintained throughout the multiple interventions involving removal of liquid covering the cells. Of particular importance was the removal of liquid between each step. Here, it was vital that the cells be disturbed as little as possible to maximize cell retention. This was achieved by leaving ~5-10 μL of liquid when aspirating. Optimization of the transfection method and treatment time involved consideration of a trade-off in intra-assay variance and assay signal: the longer the assay (i.e. standard transfection with a 24 hour treatment time) the higher the signal and variability, and vice versa. The optimization steps resulted in the 16-hour batch transfection protocol, which reduced the assay to a 3 day rather than a 4 day procedure. The optimized protocol produced an adequate degree of sensitivity, linear range of detection, and consistency and reproducibility based on acceptable inter- and intra- assay variation.

4.2. ACTIVE CHEMERIN IN OBESITY

An individual's weight status is a simple method of assessing metabolic health and the likelihood for developing obesity-associated disease. There are several ways of measuring body fat. BMI is the most widely used and there is a large existing body of knowledge that links BMI to various diseased states. BMI has been most commonly used to stratify subjects in previous human chemerin studies[74, 85, 106, 110]. Thus, it was clinically relevant to recruit our subjects based on BMI and also valid for comparisons of our data to other studies that used BMI. A key assumption of the BMI is that it attributes variability in weight at a fixed height to changes in fat mass. This disregard for body fat distribution and other sources of mass such as muscle and bone has led to criticism of BMI as a diagnostic tool for assessing if a person is overweight or obese. A great point of contention with BMI is that it has the potential to categorize athletes who have a build-up of lean mass as overweight, or that it may consider individuals belonging to certain cultures or age groups normal weight due to overestimation of lean mass. For example, BMI can be misleading in elderly people, who typically see a significant loss of muscle and bone mass[111]. We were cognisant of the limitations of BMI and thus included WHR measures to determine how chemerin corresponds to central obesity, which to date relatively few recent studies have done[112, 113].

As noted by Rourke et al.[53],the large range (0.188-15.63 nM)[87, 94] and variation in reported circulating total chemerin levels can likely be attributed to a lack of standardized methods for quantifying chemerin. These studies often differ in multiple respects that may affect measures of circulating chemerin concentrations. These include detection methods and sampling location and subject population factors such as disease status and

medication use and genetic, environmental, and cultural variance. Overnight fasting measures of total chemerin were included to compare our results to previously published data and to provide a baseline for evaluating post-feeding changes in plasma and serum chemerin concentrations. We found that total chemerin at baseline was 53% higher in plasma and 64% higher in serum of obese vs. normal weight humans. These increases are slightly higher than previous reports where subjects were grouped in a similar manner to our study[114, 115]. Total chemerin levels measured in our study (10.4-17.0 nM)were comparable to the concentrations typically reported in previously published work[78, 85, 90, 91, 94, 98, 115, 116].

Our research adds to the existing body of knowledge by including measures of chemerin over multiple time points. From this data we conclude that total chemerin remains relatively stable during early daytime hours. This is consistent with two other human studies, which found no diurnal variation in chemerin levels[87, 117]. Thus, a baseline sample after an overnight fast provides a good representation of total chemerin concentration throughout early daytime hours. In our study, total chemerin did not significantly change in the post-prandial period despite the large increase in insulin. This contrasts with *ex vivo* study by Tan et al. that found increased total chemerin secretion from human adipose tissue explants as a result of prolonged insulin infusions[87]. However, Chamberland et al. demonstrated that total chemerin levels decreased ~42%in women after a 72 hour fast relative to subjects in an isocaloric fed state suggesting that a longer fasting period than was used in the present study is required to show a difference between fasting and fed state chemerin levels. Interestingly, though Chamberland found a drop in fasting chemerin levels that could theoretically be explained by the corresponding

drop in insulin levels, subjects in an isocaloric fed state showed constant chemerin concentrations every hour over 24 hours. This is consistent with our results, which implicate against circulating chemerin being dynamic with respect to feeding *in vivo*. However, similar to our study, Chamberland et al. had a small subject population (n=6) and subjects in an isocaloric fed state never had to undergo a long fast(subjects had snacks at 22:00 and breakfast at 08:00 each of the 4 study days meaning a 10 hour fast at most). A recent study in Wistar rats found chemerin mRNA expression in perirenal WAT was approximately 5-fold higher and serum chemerin 3-fold higher in animals fed *ad libitum* for 48 hours after a 72 hour fast compared to fasted animals that were not refed[118]. This is suggestive of a potential difference in chemerin response to feeding between species, though this group did incorporate a much longer fast prior to feeding than did the present study.

Our data are the first to show that overnight fasting plasma and serum active chemerin concentrations are higher in obesity. This novel finding could indicate that obese people produce more active chemerin. An alternative interpretation of this observation is that active chemerin in obese humans is more stable or degraded less rapidly than in normal weight humans. A third possibility is that a combination of increased active chemerin production and reduced degradation are occurring. This interpretation stems from the chemerin concentrations we measured being reflective of the balance between production and degradation of active chemerin. Following feeding, the pattern of plasma active chemerin is similar to total chemerin with a more visible period of oscillation in the immediate post-prandial period that appears opposite in obese vs. normal weight. Our results of active chemerin contrast to those in Tan's study, which found that a

hyperinsulinemic clamp raised plasma total chemerin[87]. The difference in results may be explained by a prolonged duration of elevated insulin exposure in the study by Tan et al., which could allow enough time for the up-regulation of chemerin gene expression and secretion to occur versus our study in which there was only a transient insulin increase following feeding.

A similar interpretation could be drawn (as described above) if subjects were stratified as normal weight or obese by WHR. What was interesting; however, was that absolute increase in plasma active chemerin in the obese compared to the normal weight group was larger when WHR (3.60 nM) was used compared to when BMI (2.35 nM) was used. Moreover, there was a stronger correlation between active chemerin and WHR compared to active chemerin and BMI. These results indicate that increased plasma active chemerin in obesity is more strongly linked to an increase in visceral adiposity versus total adiposity. Furthermore, this also suggests that visceral adipocytes could be the more important source of altered active chemerin in obesity. In this respect chemerin may be similar to other adipokines including dipeptidylpeptidase-4, retinol-binding protein 4, interleukin-6, plasminogen activator inhibitor-1, resistin, adiponectin and TNF that display differential release form visceral compared to subcutaneous adipose depots and circulating concentrations that are strongly associated with visceral fat [119-121].However, the possibility that chemerin production and secretion is adipocyte depot-dependent has not been thoroughly investigated in humans. Supporting this interpretation, one human study found a marginal but significant elevation in total chemerin secretion from visceral compared to subcutaneous adipose tissue[120], while Tan et al. found higher chemerin mRNA expression and media concentration of total chemerin in omental

adipose tissue explants compared to subcutaneous adipose tissue explants in women with PCOS[87].

GPCR activation generally stimulates broad networks of signalling pathways with distinct biological responses that for most receptors is mediated by both G proteins and β-arrestins. Standard agonists and antagonists activate or inactivate these signalling networks in their entirety. However, biased ligands selectively engage some signals while ignoring or even inactivating other signals mediated by the same receptor[122].To date, chemerin$_{155}$, $_{157}$,$_{158}$, and $_{163}$ have been identified in plasma[54, 60]. These isoforms presumably exist in serum as well as the unknown cleavage products of factors XIIa and VIIa[54]. It is theoretically possible that different chemerin isoforms present in plasma and serum could act as biased ligands of CMKLR1. However, there is little information presently known regarding the signalling pathways of CMKLR1 (especially as they pertain to individual isoforms). Thus, we are very limited in our ability to attribute specific biased signalling pathways to the chemerin isoforms present in human plasma and serum. What is known is that the CMKLR1 bioassay used in our study operates through human chemerin$_{157}$ activating CMKLR1 and subsequent recruitment of β-arrestin2. β-arrestin2 is known to mediate the endocytosis of GPCRs by down-regulating the activated receptors ensuring that they are degraded or recycled within the cell, but can also mediate some biological effects[123]. In our assay system the increased active chemerin concentrations in obesity are within the linear range of signalling through this path, suggesting the potential for obese humans to have desensitized chemerin/CMKLR1 signalling and subsequently a suppression of downstream processes that are mediated by this signalling. These could include inflammation, and cell and proliferation and

differentiation that are linked to obesity and related diseases. Similarly, Rourke et al.[53] previously proposed the potential for chemerin/CMKLR signalling desensitization exists in obesity as a result of studies showing that chemerin treatment at low doses in human adipocytes and endothelial cells stimulated ERK1/2 phosphorylation, but not at higher doses[42, 46].

The other mechanism of CMKLR1 signalling involves inhibition of cAMP accumulation through the G_i class of heterotrimeric G proteins and seems to be activated by lower chemerin concentrations[52]. Based on the signalling-mechanisms of our bioassay we cannot make any inferences regarding the impact of obesity-related changes in active chemerin on the cAMP-signalling pathway.

Active/total chemerin ratios are important because they provide insight into the relative amount of chemerin processing taking place. This becomes particularly useful when studying chemerin function in disease: elevated active/total chemerin ratios in certain diseases may implicate a pathological role played by chemerin in specific diseased states. This concept is highlighted in Zhao's study, which observed a small increase in total chemerin in synovial fluid compared to plasma for arthritic humans, but a substantially larger increase in chemerin$_{157}$ and $_{158}$[60]. Furthermore, active/total chemerin ratios can provide insight into the mechanisms leading to increased active chemerin. Circulating levels of elastase, tryptase, and cathepsin K are all elevated in obesity, supporting the potential for increased active chemerin production in obese humans[124-126]. The obesity-related inflammatory mediator TNF also increases tryptase and elastase production by adipocytes, which are the enzymes required for conversion of prochemerin to chemerin$_{157}$ (the most active isoform)[56].It was originally hypothesized that in obese

humans the presence of low-grade inflammation would activate inflammatory cascade enzymes leading to elevated plasma active chemerin concentrations. The similar plasma active/total chemerin ratios observed in normal weight and obese humans suggest that the increased plasma active chemerin concentration in the obese humans is not attributed to increased extracellular processing of prochemerin to active chemerin. A similar notion is supported by animal studies in which mice treated with TNF showed an approximately 2-fold increase in plasma total chemerin and active chemerin[72]. Taken together, the mouse study and our human data suggest that secretion of prochemerin is the rate-limiting step in determining active chemerin concentrations and once prochemerin is secreted a constant percentage is activated to chemerin. The similar plasma active/total chemerin ratios argues against increased stability or reduced degradation of active chemerin in obese versus normal weight humans. If this was the case the active/total chemerin ratio should have increased.

The coagulation factors (XIIa, VIIa) are potent processors of prochemerin[54] and are elevated in obesity[127, 128]. Thus we also hypothesized that activation of these factors during blood clotting would lead to increased active chemerin concentrations in serum compared to plasma. The higher active/total chemerin ratios in serum compared to plasma support this hypothesis. The significantly lower active/total chemerin ratio in the obese group compared to normal weight group in serum suggests that there could be impaired processing of prochemerin to active chemerin during blood clotting in obese humans. This lower active/total chemerin ratio could potentially be attributed to the known association between obesity and impaired platelet function[129, 130]:though the biological mechanism remains unresolved, platelets in obese humans are more reactive.

Platelets store and secrete chemerin (both inactive and active isoforms[57]),and so their dysregulation could impact chemerin processing in the serum of obese humans. Similar platelet counts between groups indicate that this notion is unrelated to changes in absolute platelet number in the obese subjects. Instead, platelet degranulation as it relates to chemerin may be dysregulated in obesity. Alternatively, it is possible that the hepatic-synthesized clotting factors XIIa and VIIa that process prochemerin could be altered. However, we did not measure these proteases to determine if they were altered in the obese compared to normal weight subjects in our study.

The complete blood chemistry profile revealed that monocytes (both absolute count and % of all white blood cells (WBCs)) were significantly lower in the obese group and negatively correlated with active chemerin and total chemerin. While there are conflicting reports regarding CMKLR1 expression on circulating monocytes[63, 64, 131], in vitro human monocyte-derived dendritic cells express CMKLR1[52]. However, it is not clear in our study how reduced monocyte counts in obesity relate to increased active chemerin production or if active chemerin somehow influences monocyte levels. One of the main functions of chemerin/CMKLR1 is cell migration, so it is possible that WBC distribution could change in the presence of elevated chemerin. Whether this alteration in WBC composition is a result of elevated chemerin or vice versa is unknown.

4.3 STUDY LIMITATIONS

The small number of subjects that participated in our proof of principle study limited our interpretation of the time-dependent effects on active and total chemerin

concentrations in the post-prandial period. This limitation was overcome by grouping multiple measures from each subject and allowing for a between group comparison of active and total chemerin. The small sample size precluded a correlation analysis of active and total chemerin with metabolic parameters at baseline and the comparison of these results to previously published studies. All subjects in the present study were female, so we do not know if the same effects would be observed in males as there are conflicting studies reporting circulating chemerin concentrations to be higher in a specific gender[115, 132-134]. Additionally, we do not have enough subjects to assess age as a factor and examine how it impacts active chemerin concentrations. Low hemoglobin and mean corpuscular volume and high red cell distribution width (a measure of the variation in red blood cell volume) in the obese subjects suggest mild anemia due to iron deficiency[135]. This could have influenced our results, but as there are no known associations between hemoglobin and chemerin a potential confounding effect is unlikely. Subject 8showed relatively low active chemerin despite a high BMI. This individual had exceptionally high insulin (295 pM) and CRP (14.8 mg/L). It remains unclear if these outliers had any influence on the low active chemerin in this subject. Our data support that white adipose tissue (specifically visceral white adipose tissue) is primarily responsible for obesity-associated increases in active chemerin. However, elevated total chemerin in the hepatic vein compared to the portal vein indicate that the liver is another source of circulating prochemerin[42, 52, 85].Thus it is possible that changes in the liver production and secretion of prochemerin contribute to increased circulating active chemerin levels in our study. Arguing against this, TNF treatment in mice did not increase secretion of active chemerin in primary mouse hepatocytes whereas

it did in primary mouse adipocytes[72]. From this, it was concluded that hepatocytes likely did not contribute to the elevated serum active chemerin concentration after TNF treatment in mice. It has been proposed that CMKLR1 is activated by another ligand, resolvinE1[136], which is an anti-inflammatory mediator derived from the essential fatty acid eicosapentaenoic acid (EPA).ResolvinE1 is only formed in the presence of acid EPA and aspirin[136]. Given that our subjects were not taking aspirin, resolvinE1 activation of CMKLR1 is not likely to be impacting the results of our study.

Another limitation of our study is that it offered no insight into the presence of specific active isoforms of chemerin. The CMKLR1 bioassay is capable only of quantifying chemerin$_{157}$ equivalents in samples. As such, we cannot determine the specific chemerin isoforms that are elevated in obesity and if any CMKLR1-biased signalling occurs. The use of isoform specific antibodies as could be a helpful tool in discerning which isoforms comprise the overall active chemerin concentrations we observed; however, these are not currently available to us.

4.4 FUTURE DIRECTIONS

As we consider the therapeutic utility of chemerin, it becomes apparent that studies specifically examining chemerin isoforms as they pertain to CMKRL1 signalling pathways, biological functions, and disease pathology are vitally needed. The optimization of the CMKLR1 bioassay to quantify active chemerin concentrations in human plasma and serum has significant implications moving forward. The sensitivity offered by this assay (approximately 1.5-4 fold change above baseline at 100 pM) shows

promise for its potential usage to quantify active chemerin in situations where active chemerin is significantly lower than the concentrations observed in our study. The optimization for use with human samples provides the potential for the use of the CMKLR1 bioassay as an experimental tool for future human chemerin studies and as a diagnostic tool to measure active chemerin as a biomarker of human disease.

One of the key functions of chemerin is that it acts as a chemoattractant for different immune cells like macrophages and lymphocytes. Therefore, one mechanism to determine if elevated active chemerin in obesity is linked to function is by assessing if the migration of CMKRL1-expressing cells towards the chemerin-containing plasma or serum from obese humans is greater than in normal weight humans. An initial test with a migration assay that used CMKLR1-espressing L1.2 B-lymphoma cells showed very little migration, even in the samples with the most active chemerin as determined by the CMKLR1 bioassay. This could be due to an assay incompatibility resulting in plasma or serum harming the cells, as much fewer cells were observed under the microscope after adding the samples compared to the control. To overcome this, we will are-attempt the assay following heparin-sepharose purification of chemerin from plasma and serum[54].

Given the small sample size of our clinical study, it is imperative that further human chemerin research is done to verify our results and in other clinical populations where chemerin may be important. Future studies in broader human populations could characterize the impact of gender and age on active chemerin concentrations. This becomes especially important when considering chemerin as a therapeutic target for treating inflammation, insulin resistance, and other obesity-associated comorbidities. In addition to its negative contributions in metabolic and inflammatory dysregulation,

chemerin plays a vital role in normal metabolic and immune function[42, 66, 137]. Therefore, pharmacotherapy that targets chemerin or chemerin signalling must focus on re-establishing balance in chemerin function as opposed to completely removing it. Ideally, a chemerin-targeting therapeutic would eliminate dysregulated chemerin signalling while maintaining essential chemerin function. Studying active chemerin in human health and disease will provide information as to what the correct chemerin balance is.

Another direction that is currently being explored based on these results is to determine if there is differential active chemerin secretion from subcutaneous and visceral adipocytes from obese compared to normal weight human donors. These experiments will allow us to demonstrate in principle that elevated active chemerin in plasma of obese humans is linked to increased adipocyte production of active chemerin. Thus far, we have observed low secretion of total chemerin from subcutaneous human adipocytes and corresponding active chemerin concentrations that were below the lower limit of our assay sensitivity. We remain intrigued to see if visceral adipocytes secrete more active chemerin, as this would be consistent with the results from our clinical study, which showed a stronger linkage between active chemerin and WHR, a much better indicator of visceral adiposity accumulation than BMI. Overall, the results from these experiments should help us determine if white adipose depot specific alterations in chemerin production occur in obese humans.

4.5 CONCLUSION

In obesity, the endocrine function of fat tissue is altered resulting in increased circulating levels of pro-inflammatory/pro-diabetic adipokines that may contribute to the development of vascular and metabolic abnormalities. Given this association, adipokines and their receptors are regarded as very attractive targets for pharmacological treatment of obesity, CVD, and T2DM. We have satisfied the two study objectives originally put forth: 1) development of an assay to measure active chemerin in human plasma and serum and 2) utilizing this assay to show that active chemerin is increased in obese humans. Based on these results, I recommend that future studies of active chemerin classify obesity with relation to WHR and BMI. While I do advocate for measures of active chemerin, the fact that the plasma active/total chemerin remains constant in otherwise healthy obese people indicates that a measure of total chemerin multiplied by an active/total chemerin ratio of approximately 0.4 could be used as an estimate of active chemerin if an assay to measure active chemerin is not available. Whether such an extrapolation would apply to unhealthy obese humans or those with other inflammatory diseases is unknown. Additionally, a baseline fasting measure appears suitable as an estimate of daytime total chemerin or active chemerin concentration given a minimal effect of feeding.

The cornerstone in the treatment of obesity is lifestyle modifications. More specifically, beneficial changes in diet and exercise are seen as ideal, but are not sustainable and effective over the long term. Characterizing chemerin function is an exciting and expanding field of research with the potential to provide new therapeutic targets for obesity and associated diseases.

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APPENDIX I: Tables

Table 1. Physical characteristics of the study subjects.

Subject	Height (cm)	Weight (kg)	Waist/hip ratio (WHR)	Body Mass Index (BMI) (kg/m^2)	Systolic blood pressure (mm Hg)	Diastolic blood pressure (mm Hg)	Heart rate (beats per minute)
normal weight							
3	161	56.0	0.814	21.8	116	60	72
6	168	62.3	0.841	22.1	105	73	74
1	168	65.0	0.809	23.4	117	75	78
4	184	83.7	0.773	24.7	129	80	72
Average	**170±10**	**66.9±12.1**	**0.809±0.028**	**23.0±1.3**	**117±10**	**72.0±9**	**74.0±3**
obese							
2	165	87.4	0.876	32.0	117	70	75
7	165	95.0	0.894	34.8	109	63	44
8	169	129	0.789	45.0	139	93	92
5	154	112	0.909	47.2	136	88	76
Average	**164±6**	**106±18.6***	**0.867±0.053**	**39.8±7.5***	**125±15**	**78.5±14**	**71.8±20**

The subjects within each group are organized in order of increasing body mass index (BMI). * Indicates significant difference compared to the normal weight group, as determined by unpaired t-test ($P<0.05$).

Table 2. Fasting serum creatinine, triglycerides, free fatty acids, HDL and LDL-cholesterol, insulin, CRP and plasma glucose in obese and normal weight human subjects.

Subject	Glucose (mmol/ L)	Creatinine (μmol/L)	CRP (mg/ L)	Cholesterol (mmol/L)	TG(mmol /L)	FFA (umol/L)	Insulin (pmol/L)
normal weight							
4	5.2	61	25.5	3.8	0.7	795	94
3	4.3	61	3.4	3.4	1.0	482	42
1	4.2	67	0.3	4.1	0.6	604	77
6	4.9	47	3.8	4.4	0.8	411	63
Average	**4.65±0.5**	**59.0±8**	**8.25±11.6**	**3.93±0.4**	**0.78±0.2**	**573±168**	**69.0±22**
obese							
8	6.2	49	14.8	4.5	1.4	479	295
7	4.8	61	7.0	4.0	1.7	467	59
2	4.6	67	0.3	3.9	0.9	215	48
5	6.5	58	10.8	5.8	3.4	799	70
Average	**5.53±1.0**	**58.8±8**	**8.23±6.2**	**4.55±0.9**	**1.85±1.1**	**490±239**	**118±118**
Reference	3.30-5.60	<98.0	0-5.00	2.90-5.70	0.500-1.60	100-900	<209

The subjects within each group are organized in order of increasing BMI. CRP refers to C-reactive protein, TG refers to triglycerides, and FFA refers to free fatty acids.

Table 3. WBC, RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration in obese and normal weight human subjects.

Subject	WBC (amount/L)	RBC (amount/L)	Hemoglobin (g/L)	Hematocrit (L/L)	Mean Corpuscular Volume (fL)	Mean Corpuscular Haemoglobin (pg)
normal weight						
4	5.4	4.61	139	0.432	93.7	30.2
3	6.1	3.69	119	0.353	95.7	32.2
1	9.0	4.40	136	0.396	90.0	30.9
6	8.5	4.22	127	0.370	87.7	30.1
Average	**7.25±1.8**	**4.23±0.39**	**130±9**	**0.388±0.034**	**91.78±3.6**	**30.85±1.0**
obese						
8	7.1	4.63	126	0.373	80.6	27.2
7	5.6	4.04	108	0.333	82.4	26.7
2	4.6	3.99	101	0.324	81.2	25.3
5	5.3	4.42	114	0.375	84.8	25.8
Average	**5.65±1.1**	**4.27±0.31**	**112±1**	**0.351±0.027**	**82.3±1.9***	**26.3±0.9***
Reference	4.0-11.0 10e9/L	4.10-5.10 10e12/L	120-160 g/L	0.360-0.460 L/L	77-102 fL	26.0-35.0 pg

The subjects within each group are organized in order of increasing BMI. WBC refers to white blood cells and RBC refers to red blood cells. Mean corpuscular volume is the average volume of red blood cells, and corpuscular hemoglobin is the average amount of haemoglobin per red blood cell. *Indicates significant difference compared to the normal weight group, as determined by unpaired t-test ($P<0.05$).

Table 4. Red cell distribution width, platelet count, mean platelet volume, neutrophil %, lymphocyte %, monocyte%, and eosinophil % in obese and normal weight human subjects.

Subject	Red Cell Distribution Width (%)	Platelet Count (amount/L)	Mean Platelet Volume (fL)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)
normal weight							
4	12.7	192	11.5	57.4	27.7	10.8	3.7
3	13.0	173	10.7	64.1	19.6	9.6	6.4
1	12.7	229	10.8	62.9	26.2	9.9	0.7
6	12.2	310	10.6	56.0	32.3	7.9	3.4
Average	**12.7±0.3**	**226±61**	**10.9±0.4**	**60.1±4.0**	**26.5±5.3**	**9.55±1.2**	**3.55±2.3**
obese							
8	15.3	195	10.7	69.9	20.9	7.4	1.7
7	15.4	256	12.3	54.8	35.3	6.1	3.2
2	15.0	288	9.9	43.9	46.2	7.8	1.5
5	15.5	292	9.5	60.2	30.7	5.3	2.8
Average	**15.3±0.2***	**258±45**	**10.6±1.2**	**57.2±10.8**	**33.3±10.5**	**6.65±1.2***	**2.30±0.8**
Reference	12.2-14.3 %	186-353 10e9/L	6.8-11.2 fL	44.0-79.0 %	28.0-48.0 %	1.0-9.0 %	0-5.0 %

The subjects within each group are organized in order of increasing BMI. Neutrophil, lymphocyte, monocyte, and eosinophil % indicate the amount of each white blood cell as a percent of total white blood cells. *Indicates significant difference compared to the normal weight group, as determined by unpaired t-test ($P<0.05$).

Table 5. Basophil %, neutrophil count, lymphocyte count, monocyte count, eosinophil count, and basophil count in obese and normal weight human subjects

Subject	Basophils (%)	Absolute Neutrophil Count	Absolute Lymphocyte Count	Absolute Monocyte Count	Absolute Eosinophil Count	Absolute Basophil Count
normal weight						
4	0.4	3.1	1.5	0.60	0.200	0.000
3	0.3	3.9	1.2	0.60	0.400	0.000
1	0.3	5.7	2.4	0.90	0.100	0.000
6	0.4	4.7	2.7	0.70	0.300	0.000
Average	**0.35±0.1**	**4.35±1.1**	**1.95±0.7**	**0.7±0.1**	**0.3±0.1**	**0±0**
obese						
8	0.1	5.0	1.5	0.5	0.1	0.000
7	0.4	3.1	2.0	0.3	0.2	0.000
2	0.4	2.0	2.1	0.4	0.1	0.000
5	0.8	3.2	1.6	0.3	0.2	0.000
Average	**0.4±0.3**	**3.3±1.2***	**1.8±0.3**	**0.4±0.1***	**0.15±0.1**	**0±0**
Reference	0-1.0 %	1.5-8.0 10e9/L	1.5-5.0 10e9/L	0.0-1.0 10e9/L	0.0-0.5 10e9/L	0.0-0.2 10e9/L

The subjects within each group are organized in order of increasing BMI. Absolute neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts were measured indirectly by multiplying the % of each by the total white blood cell count. *Indicates significant difference compared to the normal weight group, as determined by unpaired t-test ($P<0.05$).

Table 6. Correlations between active chemerin and total chemerin with TG, cholesterol, FFA, CRP, insulin and glucose in normal weight and obese humans.

	Active Chemerin		Total Chemerin	
	Normal Weight	Obese	Normal Weight	Obese
Glucose	-0.322	-0.740*	-0.134	0.206
Insulin	-0.413*	0.165	0.139	0.332*
Cholesterol	0.079	0.241	-0.001	-0.062
FFA	0.168	-0.161	0.128	0.061
CRP	0.057	0.240	-0.092	0.445*
TG	0.191	-0.630*	-0.248	-0.155

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Data represent the coefficient of correlation between data sets each containing 36 values. FFA refers to free fatty acids, CRP refers to C-reactive protein, and TG refers to triglycerides.*Indicates a significant correlation.

APPENDIX II: Figures

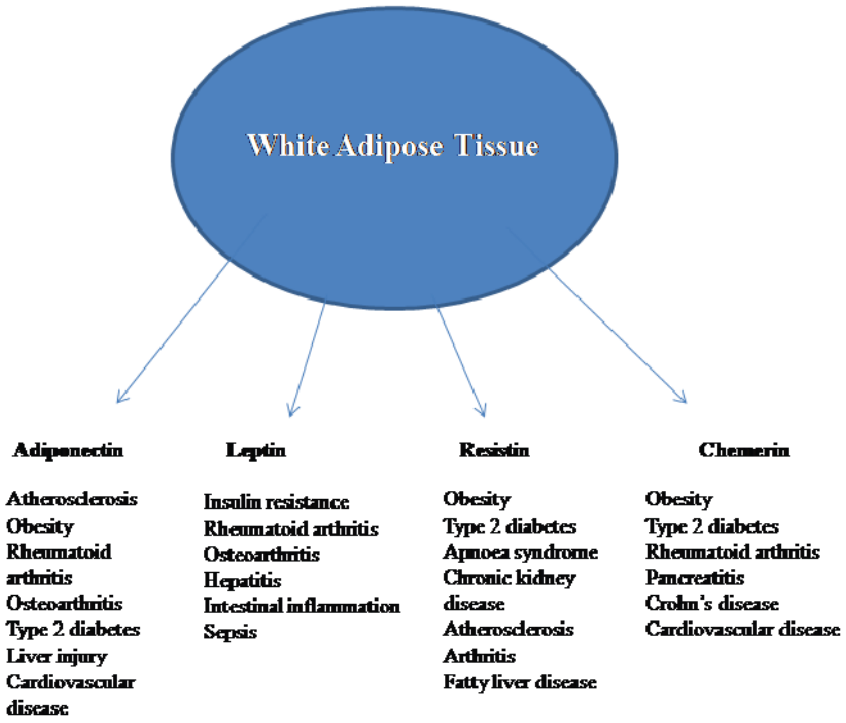


Figure 1. Representative adipokines secreted by white adipose tissue and the diseases to which they are linked.

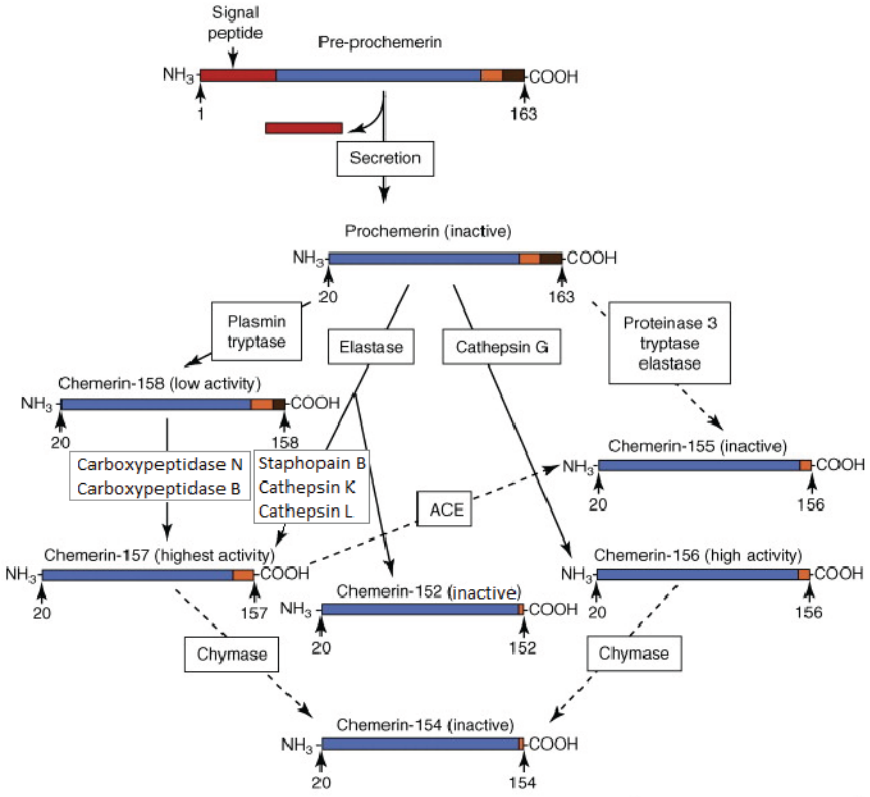


Figure 2. The mechanism of proteolytic processing of chemerin. Numbers denote amino acid sequence number.

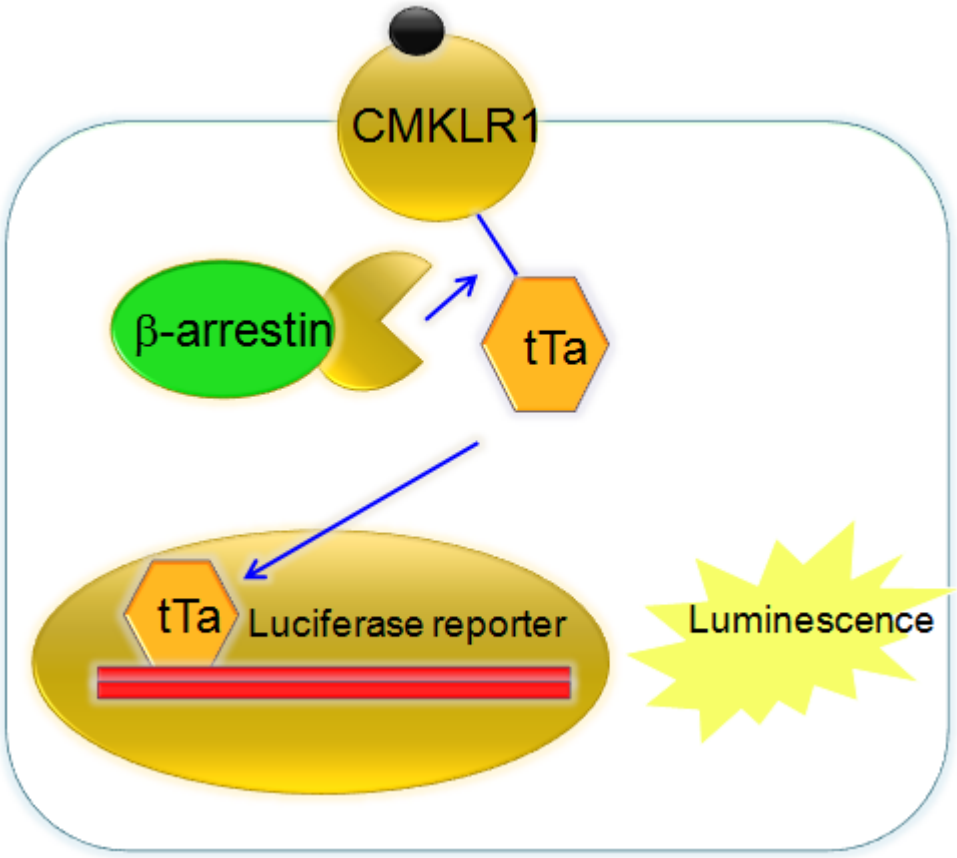


Figure 3. Schematic of the chemokine-like receptor 1 (CMKLR1) bioassay. This assay requires a transfection of a bacterial plasmidexpressing the CMKLR1 receptor fused to a transcriptional-transactivator (tTa). This DNA vector is transfected into HTLA cells, a human embryonic kidney-derived cell line containing a stably integrated tTa-dependent luciferase reporter system and a protease-tagged *β*-arrestin2 protein. When the receptor is activated by active chemerin, the protease-tagged *β*-arrestin2 is recruited to CMKLR1 where the protease cleaves off the tTa, which subsequently migrates to the nucleus of the cell and activates the luciferase reporter, causing the cells to luminesce.

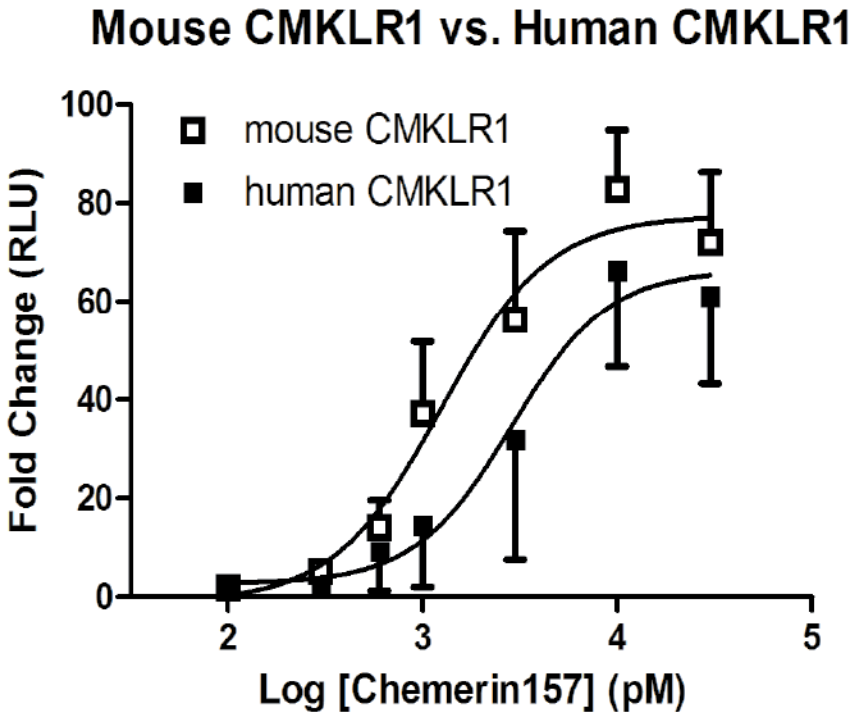
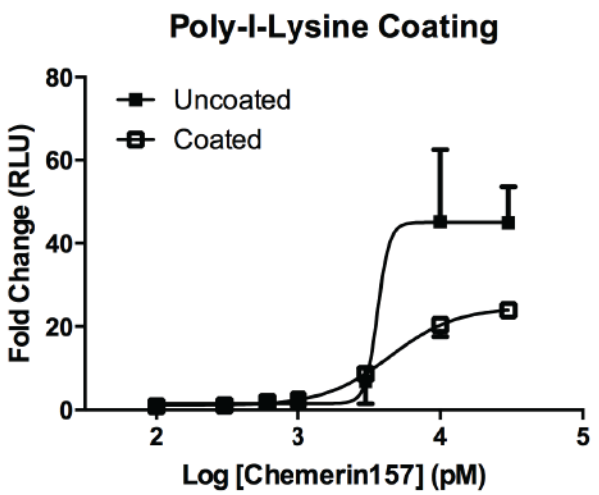


Figure 4. Assay optimization step #1: mouse and human CMKLR1 bioassay standard curves. The CMKLR1 bioassay was used with the human and mouse receptor to determine if they were differentially activated by human chemerin$_{157}$. Data points represent the means ± SEM of 4 independent experiments performed in duplicate.

A)



B)

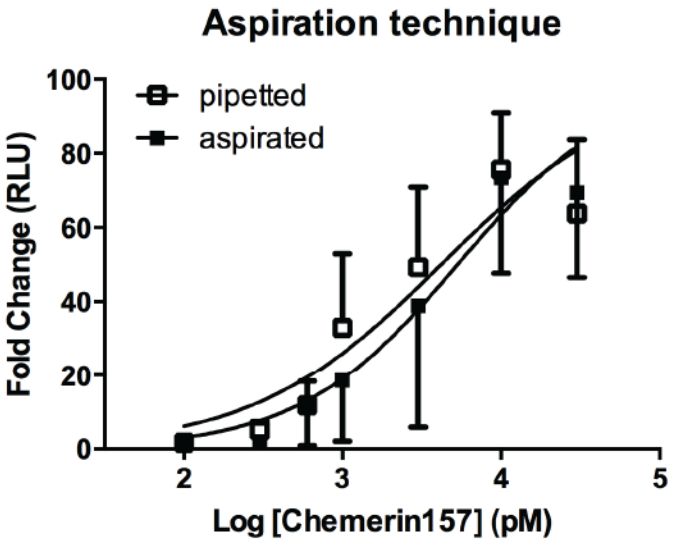


Figure 5. Assay optimization step # 2: effect of poly-L-lysine coating and Aspiration technique on CMKLR1 bioassay standard curves.
Poly-L-lysine and method of fluid removal were investigated to see if cell adherence and subsequently assay sensitivity were improved. Data points represent the means ± SEM of 2 (A) and 3 (B) independent experiments performed in duplicate.

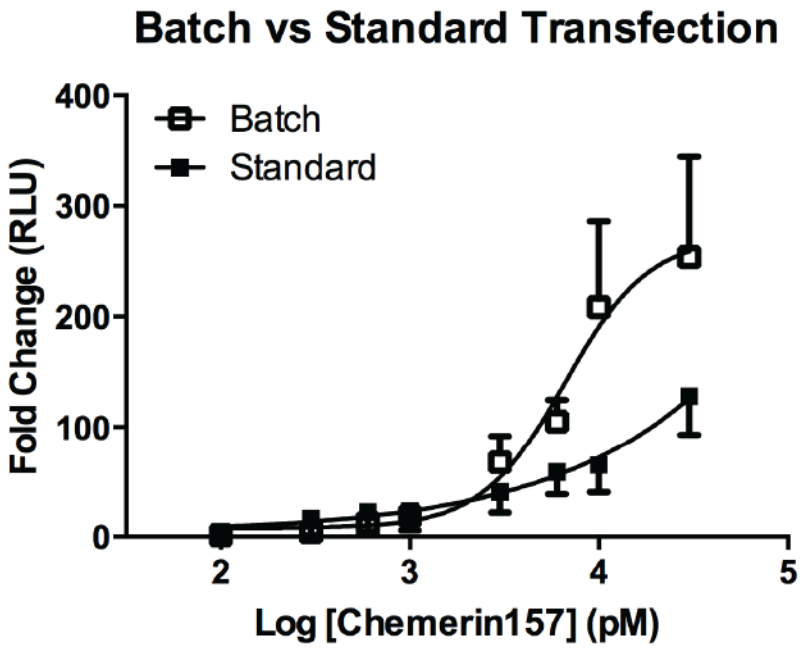


Figure 6. Optimization step #3: examining batch vs standard transfection methods in the CMKLR1 bioassay. The shorter duration batch transfection was investigated to see how it affected assay sensitivity. Data points represent the means ± SEM of 5 independent experiments performed in duplicate.

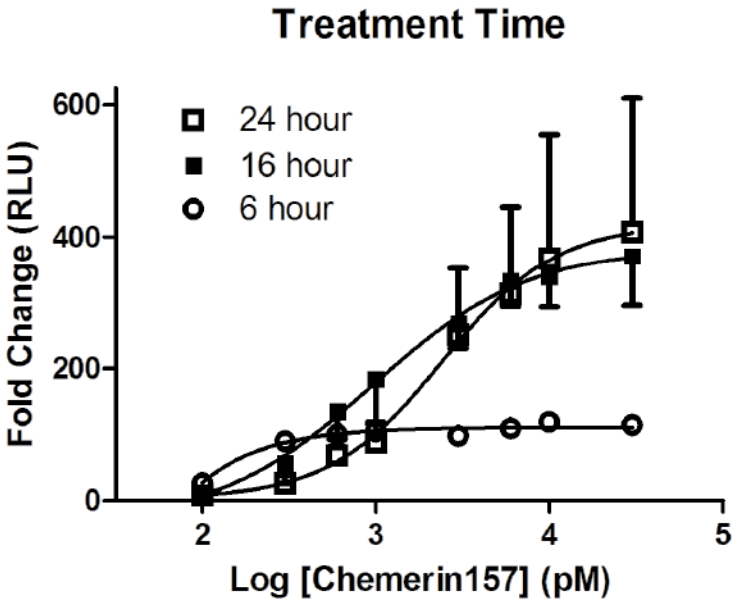
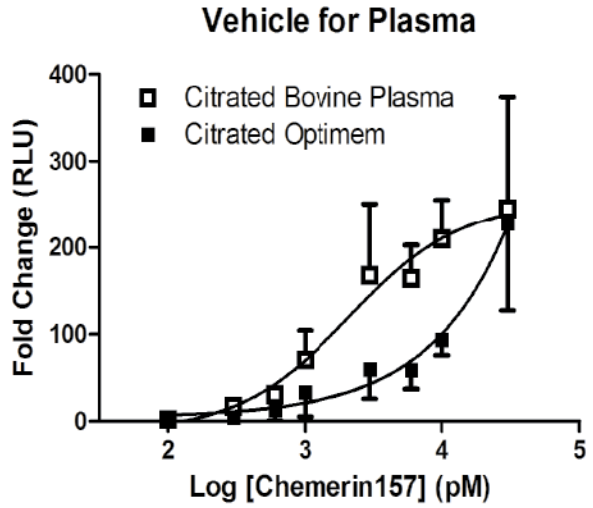


Figure 7. Optimization step #4: effect of treatment times on standard curves produced using the CMKLR1 bioassay. HTLA cells were treated with recombinant human chemerin$_{157}$ for 6, 16, and 24 hours. Data points represent the means ± SEM of 3 independent experiments performed in duplicate.

A)



B)

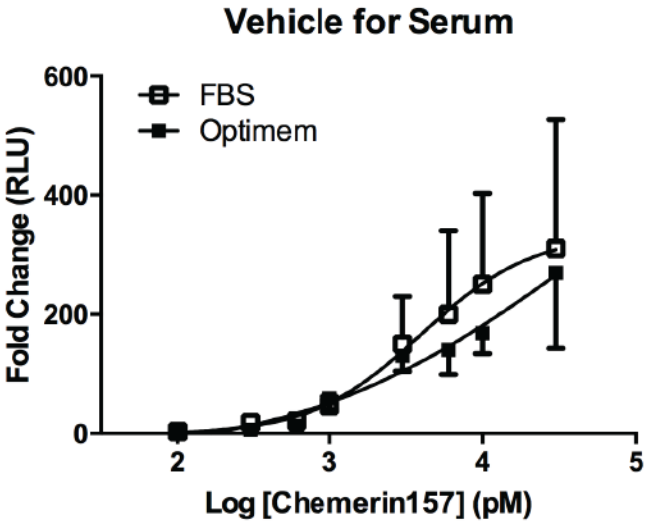
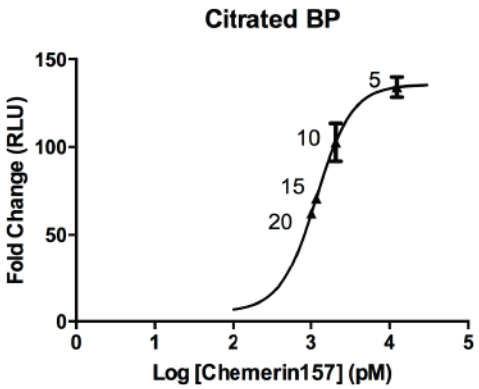


Figure 8. Optimization step #5: effect of assay diluents on CMKLR1 bioassay standard curves. Recombinant human chemerin was diluted in bovine plasma with 3.2% sodium citrate diluted 1:10 in optimem and 3.2% sodium citrate diluted 1:10 in optimem as potential vehicles for plasma (A), or in FBS and optimem as potential vehicles for serum (B). Data points represent the means ± SEM of 3 independent experiments performed in duplicate.

A)



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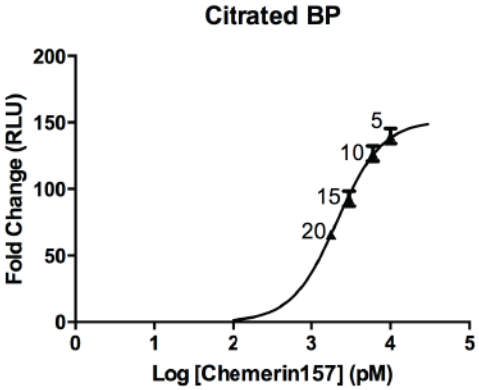


C)

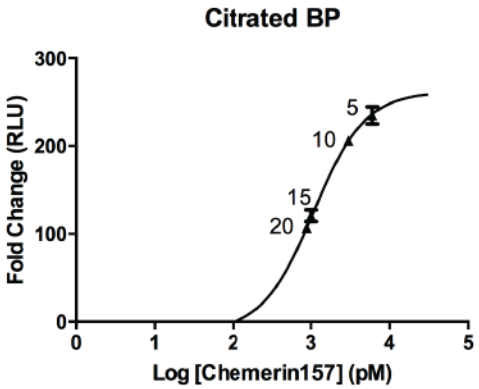
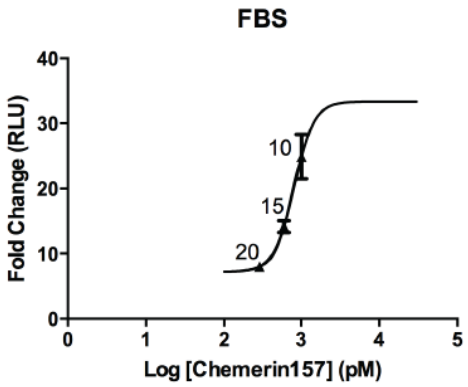
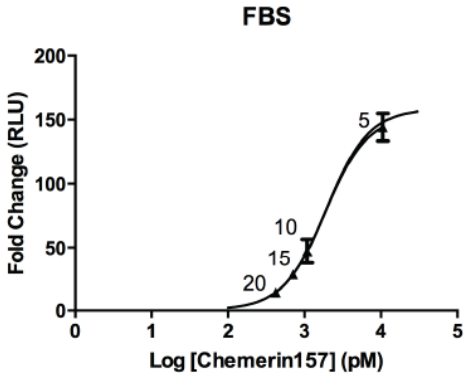


Figure 9. Optimization step #5: human plasma at different dilution factors in the CMKLR1 bioassay. Control plasma samples were diluted 1 in 5, 10, 15 and 20 in optimem. The diluent used to produce the standard curves was bovine plasma with 3.2 % sodium citrate diluted 1:10 in optimem for A) and bovine plasma with 3.2% sodium citrate diluted 1:20 in optimem for B) and C). Data points represent the mean ± SEM of an individual experiment performed in triplicate.

A)



B)



C)

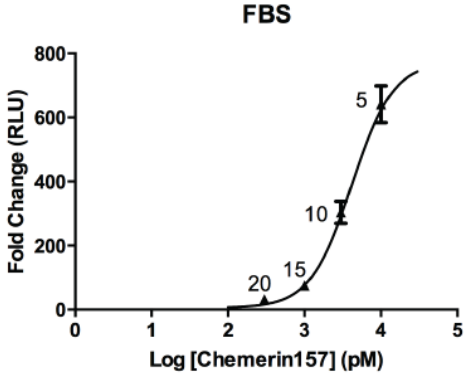
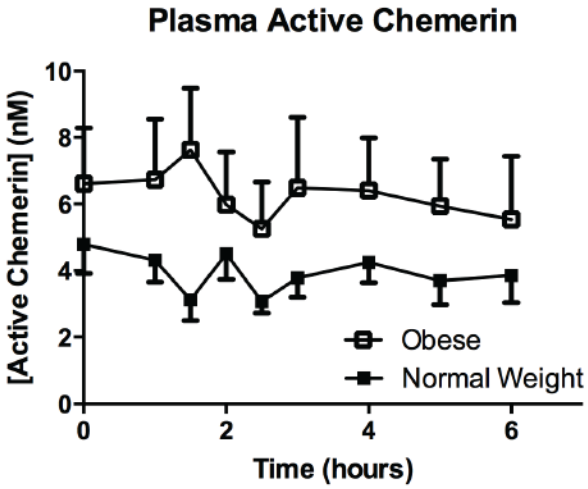


Figure 10. Optimization step #5: human serum at different dilution factors in the CMKLR1 bioassay. Control serum samples were diluted 1 in 5, 10, 15 and 20. The diluent used to produce the standard curves was FBS diluted 1:10 in optimem. Data points represent the mean ± SEM of an individual experiment performed in triplicate.

A)



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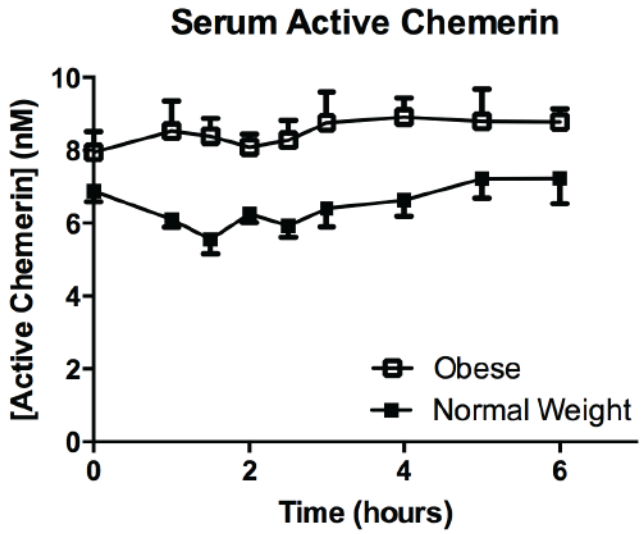
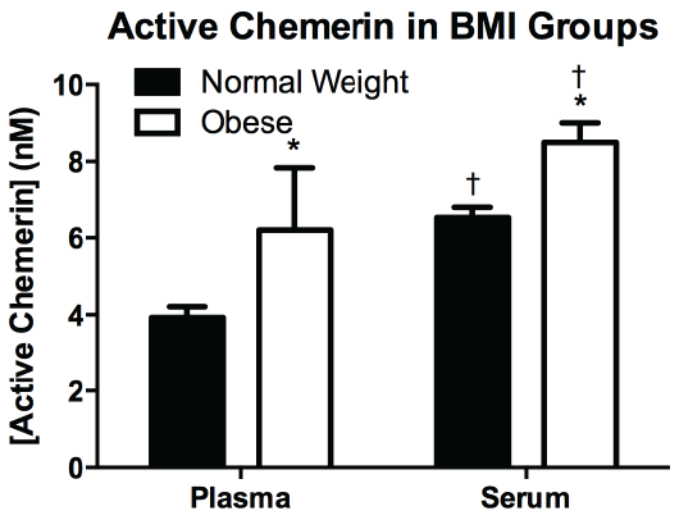


Figure 11. Active chemerin concentrations in the normal weight and obese groups in plasma (A) and serum (B). Breakfast was given at 1 hour and consumed over approximately 30 minutes. Time represents time elapsed after the first sample was collected. Data points represent the means ± SEM of 4 subjects.

A)



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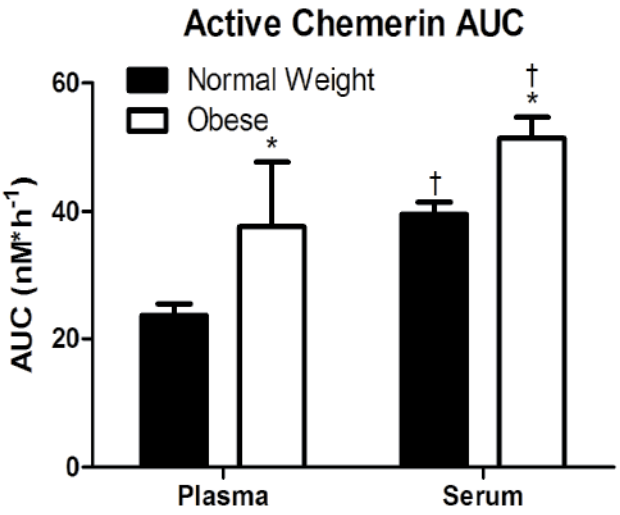

Figure 12. Active chemerin concentration and AUC in normal weight and obese humans in plasma and serum. Each bar represents the mean + SEM of 36 data points. * Indicates a significant difference compared to normal weight and † indicates a significant difference compared to plasma, as determined by a two-way ANOVA, followed by Bonferonni multiple comparison test ($P<0.05$).

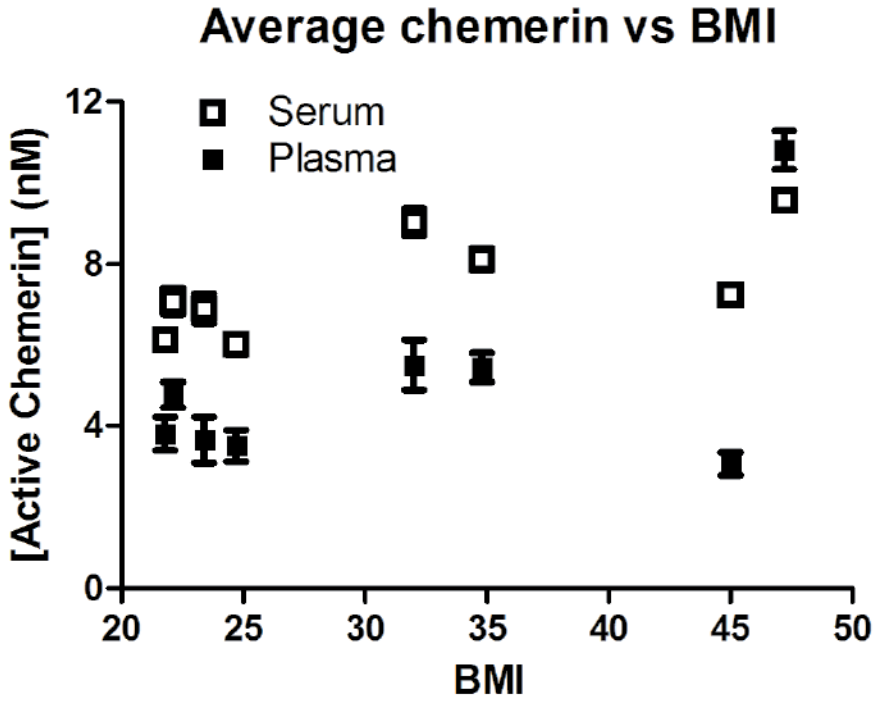

Figure 13. The association between active chemerin and BMI. Each data point represents the mean active chemerin concentration + SEM over all 9 time points.

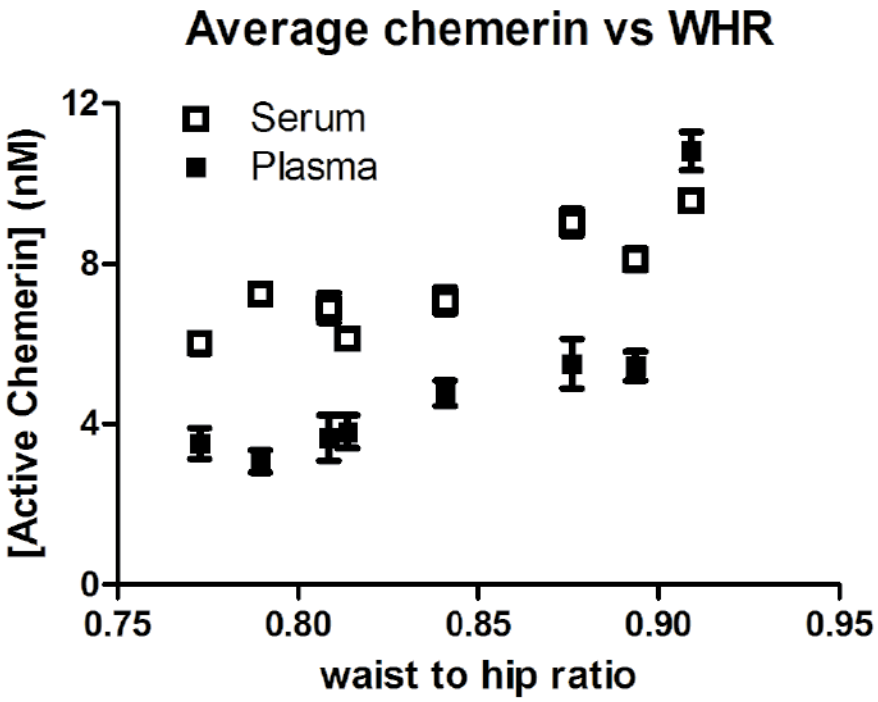

Figure 14. The association between active chemerin and WHR. Each data point represents the mean active chemerin concentration + SEM over all 9 time points.

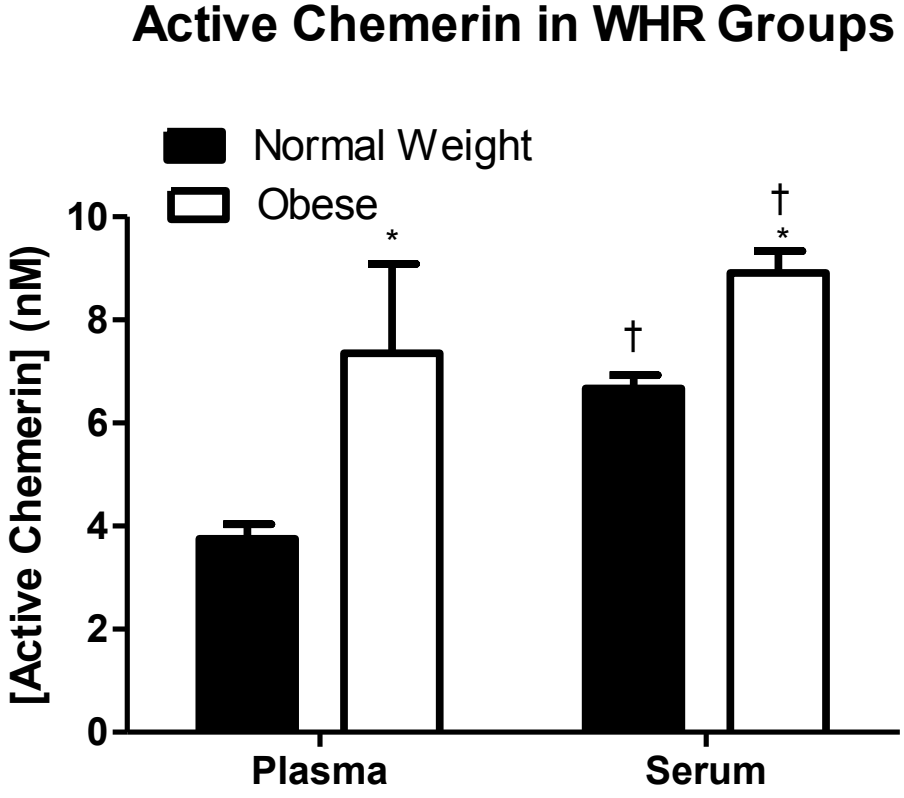

Figure 15. Active chemerin in each WHR group. Each bar represents the mean + SEM of 36 data points. * Indicates a significant difference compared to normal weight and † indicates a significant difference compared to plasma, as determined by a two-way ANOVA, followed by Bonferonni multiple comparison test ($P<0.05$).

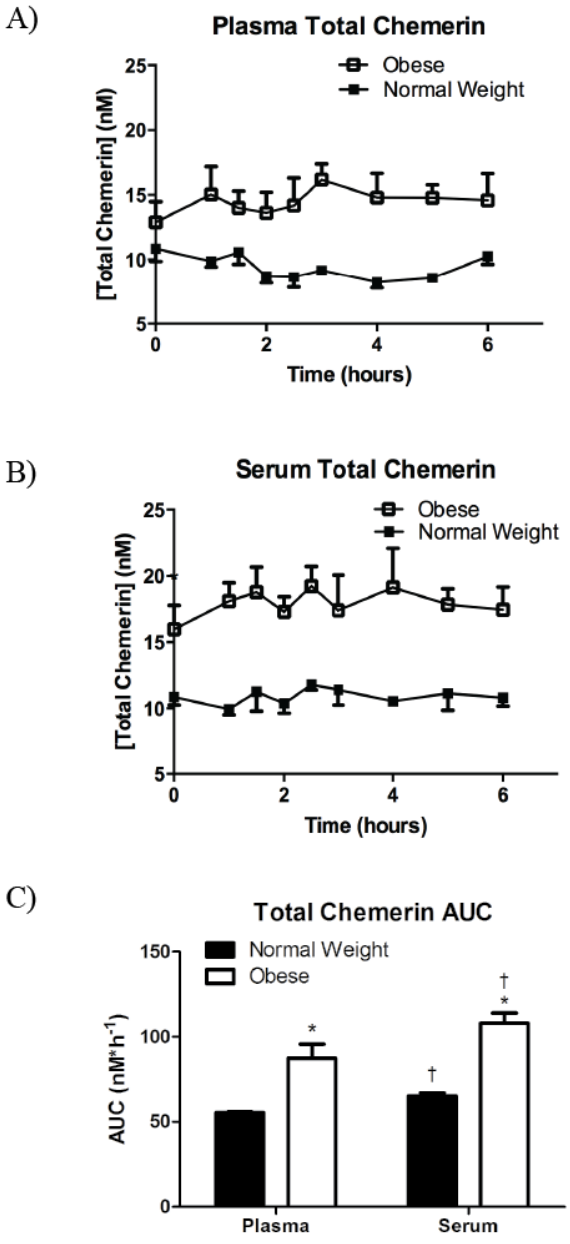

Figure 16. Total chemerin concentrations in the normal weight and obese groups in plasma (A) and serum (B) and total chemerin AUC (C). Breakfast was given at 1 hour and consumed over approximately 30 minutes. Time represents time after the first sample was collected . Data points represent the means ± SEM of 4 subjects (A and B) and each bar represents the mean + SEM of 36 data points (C). * Indicates a significant difference compared to normal weight and † indicates a significant difference compared to plasma, as determined by a two-way ANOVA, followed by Bonferonni multiple comparison test ($P<0.05$).

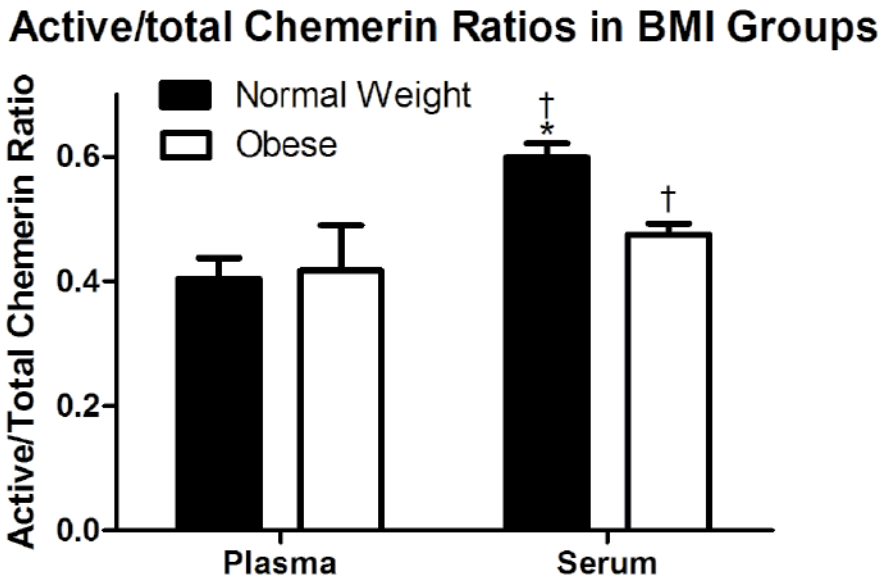

Figure 17. Active/total chemerin ratios for the normal weight and obese groups in plasma and serum. Groups are categorized by BMI. Each bar represents the mean + SEM of 36 data points. * Indicates a significant difference compared to the obese group and † indicates a significant difference compared to plasma, as determined by a two-way ANOVA, followed by Bonferonni multiple comparison test ($P<0.05$).

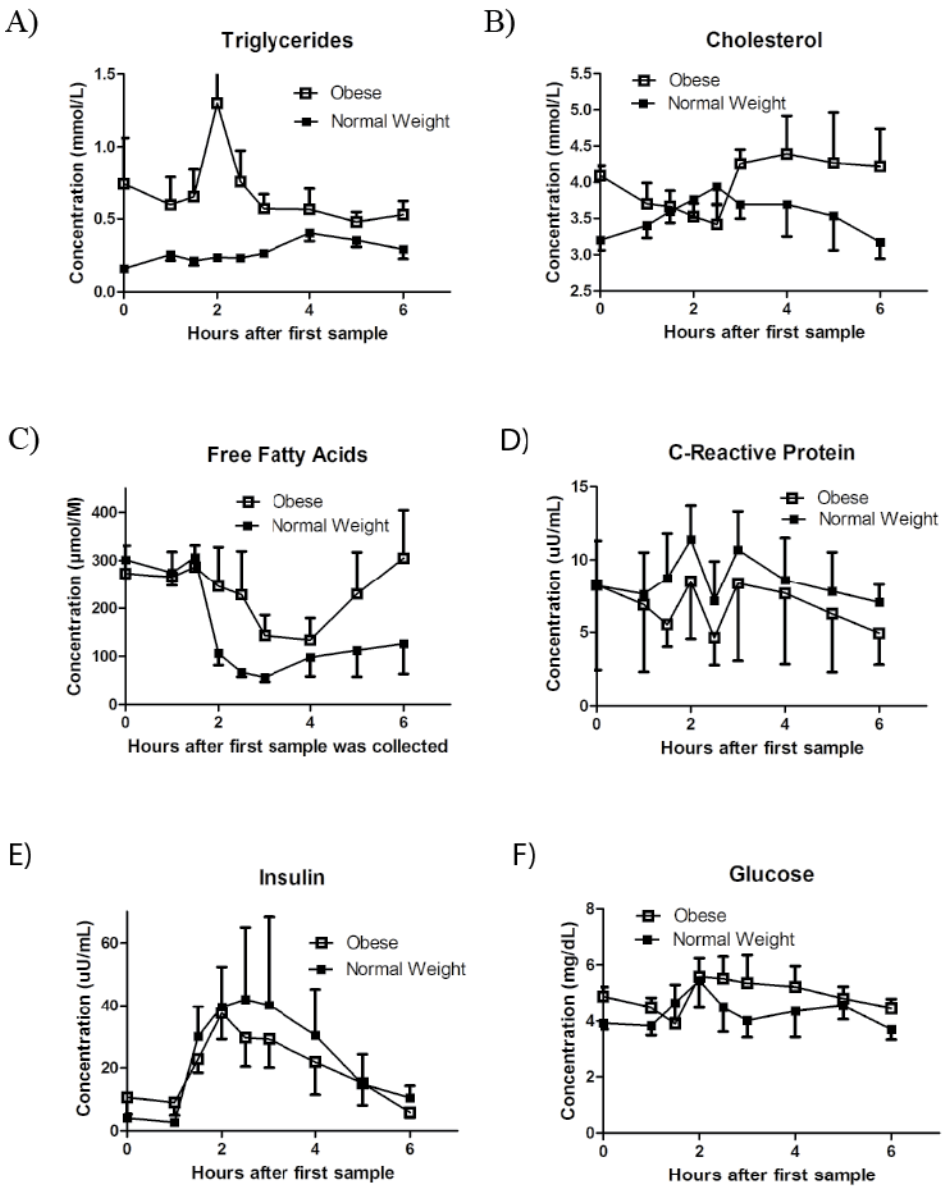

Figure 18. Triglycerides(A), Cholesterol (B), Free fatty acids (C), C-Reactive protein (D), Insulin (E), and Glucose (F) in normal weight and obese humans. Breakfast was given at 1 hour and consumed over approximately 30 minutes. Time represents time elapsed after the first sample was collected. Data points represent the means± SEM of 4 subjects.