NTNU – Trondheim
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The Effect of a balanced Diet on Serum IL-6 Concentration in obese Women.

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Abstract

Word Heath Organization (WHO) estimates that 2.8 million adults die each year as a result of obesity. Raised Body Mass Index (BMI) can lead to lifestyle diseases such as Cardio Vascular Diseases (CVD), Type 2 Diabetes (T2D) and some cancers. For many years the focus has been to reduce the total intake of fat to reduce obesity. The role of dietary carbohydrates in conjunction to lifestyle diseases has been less studied.

Obesity often leads to chronic low-grade inflammation and insulin resistance, which can lead to lifestyle diseases. Through the "food and genome" project at Norwegian University of Science and Technology (NTNU), two studies have been done to investigate how distribution of macronutrients in the diet affects chronic low-grade inflammation. The result shows that by decreasing the amount of dietary carbohydrates in conjunction to governmental recommendations the inflammatory status of the body can be improved.

Obese individuals seem to have an increased level of several cytokines, including Interleukin-6 (IL-6). The level of IL-6 has been shown to correlate with both C-Reactive Protein (CRP) and BMI, and decrease after weight loss. IL-6 can be used as a marker for inflammation, as it is a part of many inflammatory pathways in the cell.

In NTNU III, 28 females with BMI>30 were given two diets; AHC (diet A, High Carbohydrate) and BMC (diet B, Moderate Carbohydrate). The aim of this thesis is to investigate whether a balanced diet affects the levels of IL-6 in blood serum, and if the concentration correlates to CRP and BMI. Blood samples were collected at baseline, after AHC and after BMC. The concentration of IL-6 was detected in blood serum using sandwich Enzyme Linked Immunosorbent Assay (ELISA).

No significant change in IL-6 concentration was found after the diets. No significant correlation was detected between IL-6 and BMI or IL-6 and CRP. Chronic low-grade inflammation and insulin resistance are complex conditions. In order to say something about the inflammatory profile it is necessary to look at the broader picture. Chronic low-grade inflammation involves several biological compounds that need to be further investigated.

Sammendrag

WHO har estimert at rundt 2.8 millioner voksne dør som følge av overvekt hvert år. Med økt BMI øker også risikoen for livsstilssykdommer som hjerte og karsykdom, type 2 diabetes og noen krefttyper. Det har i mange år vært fokus på å minske inntaket av fett i dietten for å hindre overvekt, men hvordan karbohydrater påvirker livsstilsykdommer har det vært mindre forskning på.

Kronisk inflammasjon og insulin resistans er tilstander som kan forårsakes av overvekt og kan føre til livssykdommer. På NTNU er det gjort to studier i "mat og gen" prosjektet som indikerer at en endring i forholdet mellom næringsstoffene i kosten kan påvirke inflammasjonsstatusen i kroppen. Resultatene viser at ved å spise mindre karbohydrater og mer fett enn hva helsedirektoratet anbefaler kan man minske den inflammatoriske statusen i kroppen.

IL-6 er en av flere cytokiner som har økt konsentrasjon hos overvektige individer. Studier har vist at konsentrasjonen av IL-6 korrelerer med CRP og BMI, og blir redusert etter vektnedgang. IL-6 kan brukes som en indikasjon på inflammasjon, da cytokinet er en viktig del av flere inflammatoriske prosesser i cellene.

I NTNU III har 28 damer med BMI>30 gått på to ulike dietter; AHC (diett A, Høy Karbohydrat) og BMC (diett B, Moderat Karbohydrat). Målet med denne oppgaven er å se om et balansert kosthold i forhold til næringsstoffene i dietten påvirker nivået av IL-6 i blodserum, og om nivået korrelerer med CRP og individenes BMI. Blodprøver ble tatt før AHC dietten, mellom diettene og etter BMC dietten. Konsentrasjonen av IL-6 ble funnet i blod serum ved bruk av sandwich ELISA.

Det ble ikke funnet noen signifikant endring av IL-6 etter diettene og ingen signifikant korrelasjon mellom IL-6 og CRP eller IL-6 og BMI. Kronisk inflammasjon og insulin resistans er komplekse tilstander. For å si noe om den inflammatoriske statusen i kroppen er det nødvendig å se på flere biologiske molekyler som er involvert i kronisk inflammasjon.

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1. Abbreviations

AHC	Diet A, high carbohydrate diet
BMC	Diet B, moderate carbohydrate diet
BMI	Body mass index
CNTFRα	Ciliary neurotrophic factor receptor α
CRP	C-reactive protein
CVD	Cardiovascular diseases
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FFA	Free fatty acids
GEF	GTP exchange factor
GI	Glycemic index
Gp130	Glycoprotein 130
Grb-2	Growth-factor-receptor-bound protein 2
HRP	Horseradish peroxidase
HRP C	Horseradish Peroxidase C
hs-CRP	High sensitive c-reactive protein
IL	Interleukin
IL-6	Interleukin-6
IL-6Rα	Interleukin-6 receptor
IL-11R α	Interleukin-11 receptor
IkB	Inhibitor of kappa B

IKK	IκB kinase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LIFR	Leukaemia inhibitory factor receptor
MAPK	Mitogen-activated protein kinase
MHO	Metabolically healthy but obese
NFκB	Nuclear factor-kappa B
NTNU	Norwegian University of Science and Technology
UPR	Unfolded protein response
PAI-1	Plasminogen activator inhibitor type 1
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-4.5-bisphosphate 3-kinase
RCF	Relative centrifugal force
Rpm	Rotations per minute
SAA	Serum amyloid A
SH2	Src Homology 2
sIL-6R	Soluble IL-6 receptor
Socs	Suppressor of cytokine signaling
STAT	Signal transducers and activators of transcription
T2D	Type two diabetes
TGF-β	Transforming growth factor beta
TLR	Toll like receptor
TMB	Tetramethyl benzidine

TNFα	Tumor necrosis factor alpha
TSA	Tyramide signal amplification
WHO	World health organization

2. Introduction

2.1 Obesity and lifestyle diseases

The prevalence of obesity has doubled since the 1980 and WHO estimates that 2.8 million adults die each year as a result of obesity and overweight [2]. In 2010, 35.7% of adults in the US were overweight [3], and in England the percentage has reached 61.9 [4]. In Norway, BMI has increased over the past 20 years [5], and today around 20% of the Norwegian population is considered overweight [6].

Overweight and obesity is defined as excess fat accumulation that may impair health, and is measured by BMI (kg/m^2). BMI equal to or higher than 25 is considered as obesity and BMI equal or higher than 30 is considered as overweight [2].

Obesity is a result of an imbalance of energy, when the consumption of calories exceeds calories expended. Increased BMI is a risk factor for several noncommunicable diseases such as CVD, T2D and some cancers. CVD was the leading cause of death in the western world in 2012 [2], and the prevalence of T2D continue to rise [7]. Dietary guidelines for Americans suggest restricted calorie intake to lose weight [8] and the western world have followed the same recommendations.

2.2 Composition of dietary macronutrients

The focus of governments has been to reduce the dietary intake of saturated fat in order to reduce obesity. In Norway, the total intake of dietary fat was reduced from 40-35 E% from 1970-1990, and carbohydrates replaced the removed fat content [9]. The 2010 Dietary guidelines advisory committee states that the average American eat too much solid fat, and recommends carbohydrates as the main source of calories [8]. Both the food industry and the public population have followed the governmental recommendations given, but obesity related lifestyle diseases is still an increasing problem worldwide [10].

Dietary carbohydrates in conjunction with lifestyle diseases have been less studied. Carbohydrates is the main source of calories for the average American [8], and the Nordic nutrition recommendations states that the intake of dietary carbohydrates should be 45 E% [11].

2.3 Glycemic index and glycemic load

Glycemic Index (GI) is a relative measurement of the rise in blood glucose after a meal. Glucose is the definition standard with a GI of 100. Glycemic Load (GL) also accounts for the quantity of carbohydrates by multiplying GI with grams of carbohydrate per serving size [12].

Low GI food causes a slower rise in blood sugar and maintains energy for a longer period of time compared to high GI food [12]. Studies show a beneficial effect of diets with a low GI with respect to inflammatory markers such as CRP [13], indicating that not only the quantity of carbohydrate is important, but also the quality.

There is a current debate about the dietary macronutrient composition and the effect on inflammation. A paper published in Obesity in 2008 investigated how different meals affected the postprandial inflammatory profile after 1, 4 and 6 hours. Results showed that the type of carbohydrate digested affected the concentration of IL-6, while the type and quantity of fat showed no effect. The increase of circulating IL-6 was higher postprandial to carbohydrates with high GI compared to low GI, and a larger increase was observed in obese individuals compared to lean subjects [14]. High GI food has been shown to up regulate Nuclear Factor-Kappa B (NFκB) [15], which in turn transcribe pro-inflammatory cytokines such as IL-6 [16]

2.4 Chronic low-grade inflammation, insulin resistance and obesity

Obesity, T2D and CVD are all associated with insulin resistance and chronic low-grade inflammation [17]. Insulin resistance is the onset condition for T2D and can be caused by obesity and diet induced low-grade inflammation. Insulin resistance can occur in several tissues, and in response, β-cells in the pancreas produce more insulin. This can only occur up to a certain limit before the β-cells fails and one develops T2D [18].

There seems to be a close relationship between metabolic diseases and immune dysfunction. Obesity leads to low-grade inflammation, which again leads to insulin resistance [reviewed in [19]].

2.4.1 Chronic low-grade inflammation

Inflammation is the response to infection and tissue damage caused by pathogens or cell damage [20]. During the acute phase of inflammation IL-6, a pro-inflammatory cytokine, stimulates production of acute phase proteins such as CRP and Serum Amyloid A (SAA) to activate the immune response. During the acute phase response there can be a 10-100-fold increase of both CRP and SAA [21].

A second view of the term inflammation refers to the up-regulation of inflammatory mediators without the classical acute phase response. The literature refers to this condition as low-grade inflammation. It differs from the acute phase response because it lacks the classical inflammatory signs like swelling and raised temperature [20]. Low-grade inflammation produces a lower level of circulating cytokines [22], and is considered to be a chronic condition. Minor CRP elevation is regarded as an indicator of low-grade inflammation [20].

Adipose tissue is considered an endocrine organ that secretes adipokines such as leptin, adiponektin and other biological molecules [23, 24]. Adipose tissue also secretes pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα) and IL-6 [25-27]. The amount of secreted cytokines correlate with the adipocyte volume [28], and obesity is thus regarded as a state of chronic low-grade inflammation [29]. There is evidence of BMI correlating with both circulating levels of IL-6, TNFα and CRP [29], and that the level decreases following weight loss [30].

A chronic activation of the innate immune system and NFκB is thought to be a underlying mechanism for low-grade inflammation [31]. NFκB induce transcription of several pro-inflammatory cytokines [32], hence the high level of IL-6 in adipose tissue is most likely the source of the increased CRP concentration found in obese individuals [23].

The release of pro-inflammatory molecules from adipose tissue has been suggested to cause the metabolic complication of obesity. Obesity causes accumulation of macrophages in adipose tissue, providing the mechanism of increased release of pro-inflammatory mediators (figure 1). Adipocyte size seems to be a strong indicator of percentage macrophage accumulated in the adipose tissue [33].

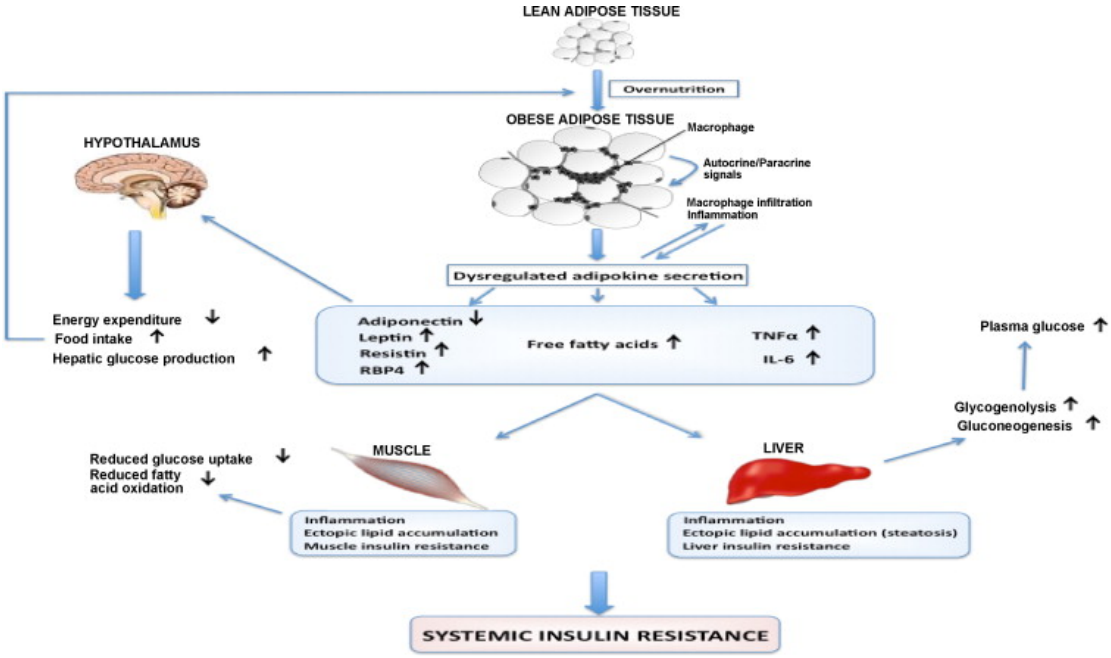

Figure 1 Expansion of adipose tissue induce accumulation of macrophages and inflammation hence causing an increase in secreted pro-inflammatory molecules such as TNFα and IL-6. There is also dys-regultation of leptin, adiponectin, resistin and retinol binding protein-4 (RBP4). Combined, these factors lead to both low-grade inflammation and insulin resistance in the muscle and liver [24].

2.4.2 Linking low grade inflammation to insulin resistance

Insulin resistance is a state where cell response to insulin is insufficient. This condition leads to an increase of circulating blood glucose, which is not stored in the liver and muscle cells [34].

Up-regulation of pro-inflammatory cytokines such as TNFα causes an increase of circulating insulin, as TNFα is reported to inhibit insulin signaling [35]. TNFα and insulin mediate inhibitory phosphorylation of the insulin receptor, and inhibit transcription of GLUT 4 that transports glucose into the cell [22].

It is widely accepted that obesity linked inflammation is a cause of errors in the insulin signaling pathway and hence a cause of insulin resistance. Many underlying molecular mechanisms have been suggested, but these mechanisms are still not entirely understood. There are several lines of evidence of different components and molecules being part of obesity linked insulin resistance [18].

Saturated Free Fatty Acids (FFA) act through Toll Like Receptor 4 (TLR 4) to induce inflammation through NFκB activation in macrophages, adipocytes and hepatocytes [36]. Endoplasmic Reticulum (ER) stress is shown to link obesity induced inflammation and insulin resistance. ER stress causes unfolded proteins, which leads to an Unfolded Protein Response (UPR). UPR in macrophages and adipocytes cause activation of inflammatory pathways signalled though IκB kinases (IKK) and c-Jun N-terminal Kinase (JNK) [20, 37]. Activated JNK further activates Activator Protein-1 (AP-1), which function as a transcription factor for several pro-inflammatory cytokines (figure 2) [38].

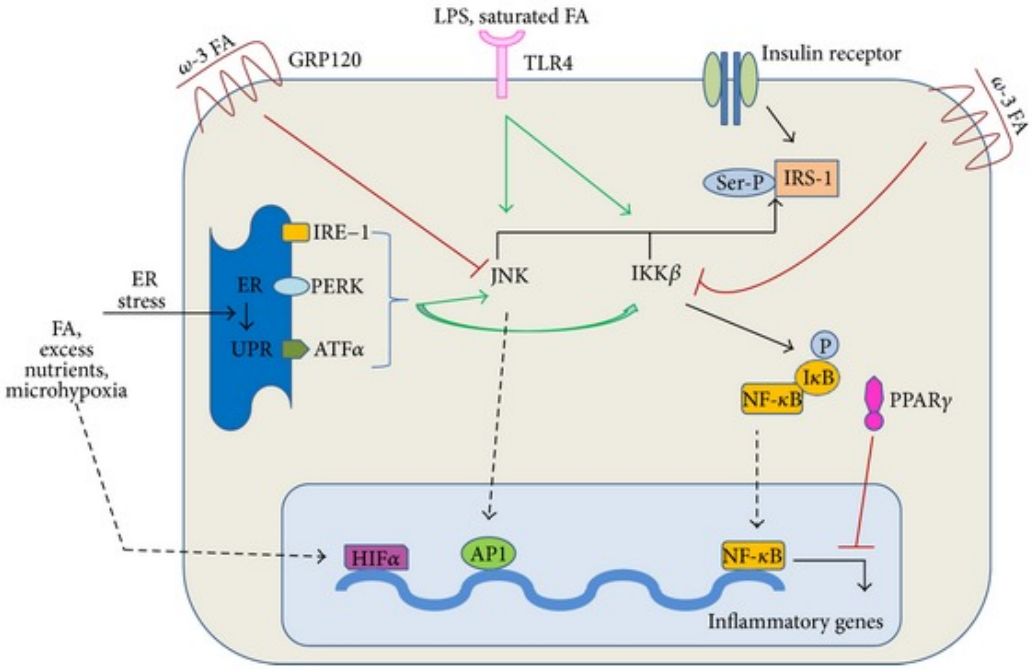

Figure 2 Several signalling pathways promote or inhibit inflammation and interact with insulin signalling. ER stress causes activation of JNK and IKKβ, as do FFA though TLR4, which leads to transcription of pro-inflammatory cytokines and inhibition of the insulin-signalling pathway. [19]

2.5 Cytokines

Cytokines are small soluble proteins produced by cells of the immune system and several other types of cells. They can alter the behaviour of other cells, or the secreting cell itself. Most cytokines produced by T-cells are called interleukins (IL) [39].

2.5.1 Interleukin-6

The human IL-6 protein is a single polypeptide chain containing 185 amino acids residues. It is organized in four alpha helices linked together by loops and mini-helices (figure 3) [40]. IL-6 is produced by several cell types, like T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, astrocytes, bone marrow stroma cells and meningeal cells (figure 4) [41]. Both muscle and adipose tissue are known to secret IL-6 [25, 42]. In lean healthy individuals, serum concentration of IL-6 is normally <3-4 pg/ml [43].

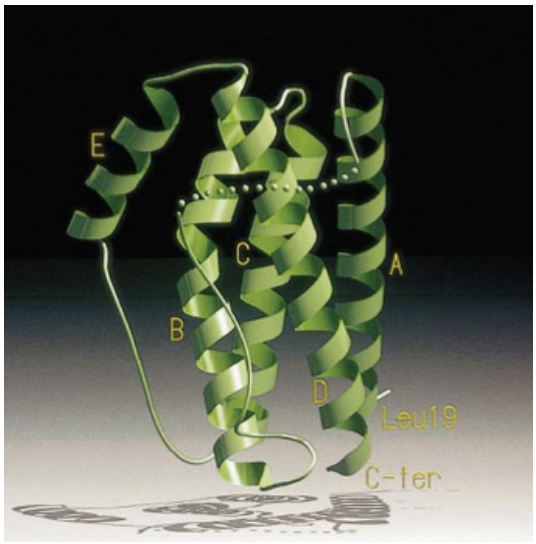

Figure 3 Crystal structure of IL-6. The main alpha helices are labelled A, B, C, D and E [40]**.**

IL-6, together with other cytokines, plays a crucial role in the acute-phase response to infection. In the liver IL-6 activates acute phase proteins like CRP and induces fever [39]. IL-6 is a pleiotropic cytokine that activates the immune system and is part of the regenerative processes, regulation of metabolism, bone homeostasis and several neural functions (figure 4) [44].

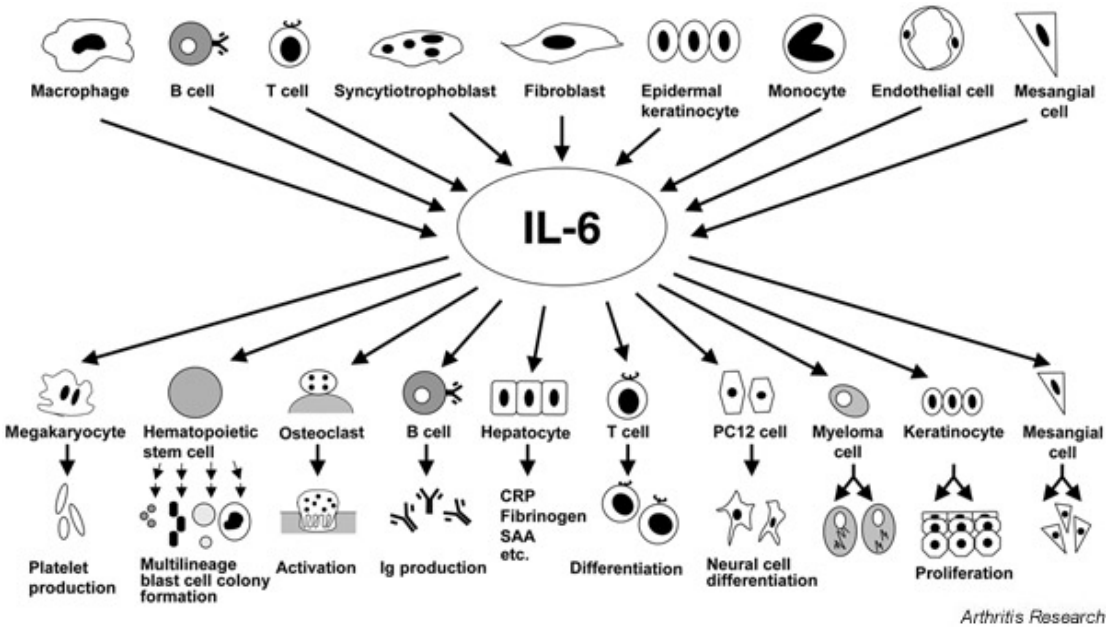

Figure 4 Cell types that produce IL-6 (on top), and the biological activities of IL-6 on target cells (bottom) [45].

2.5.2 IL-6 signalling

IL-6 type cytokines activate the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) signalling pathway [46] and Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinases (MAPK/ERK) cascade through receptor glycoprotein 130 (gp130) [47, 48]. Receptor recognition of IL-6 type cytokines can be subdivided into non-signalling α receptors (IL-6 Receptor α (IL-6Rα), IL-11 Receptor α (IL-11Rα) and Ciliary Neurotrophic Factor Receptor α (CNTFRα)) and signal transducing receptors (gp130, Leukaemia Inhibitory Factor Receptor (LIFR) and Oncostatin M Receptor (OSMR)). The signal transducing receptors is associated with JAKs and is phosphorylated in response to ligand binding [48]. All receptor complexes compose of at least one gp130 molecule as shown in figure 5.

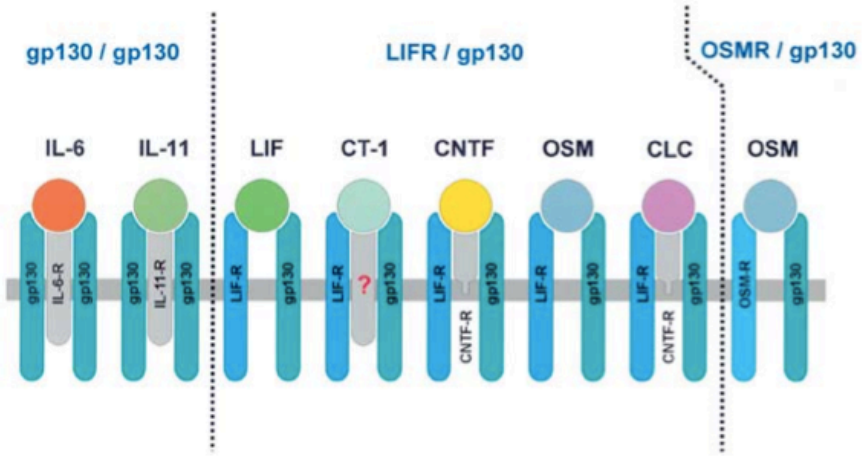

Figure 5 IL-6 type cytokines all signal through receptor complexes composed of gp130 in combination with other receptor subunits such as gp130, LIFR and ORMR [48]

IL-6 first binds to the non-signaling α receptor. This complex further binds to two gp130 molecules (figure 6) to activate the IL-6 transduction pathways including JAK/STAT, MAPK/ERK and Phosphatidylinositol-4.5-Bisphosphate 3-Kinase (PI3K) [49]. A schematic overview of the signalling pathways is shown in figure 7.

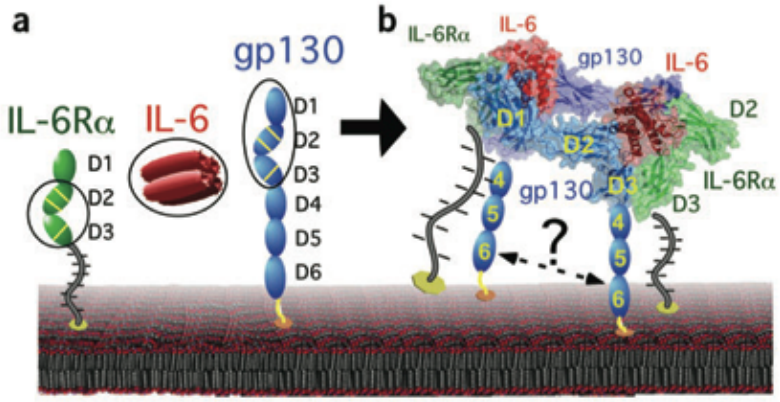

Figure 6 A: The domain structure of IL-6Rα, IL-6 and gp130 receptor. B: Schematic representation of the hexametric complex upon binding of IL-6 ligand to two gp130 receptor and IL-6Rα [50].

Binding of IL-6 to the receptor complex leads to homodimerization of the gp130 molecules [51]. JAK 1 and JAK 2 are associated with the receptor complex and trans-phosphorylate and activate each other upon dimerization. The JAKs phosphorylate tyrosine residues on the cytokine receptor, creating docking sites for STAT 1 and STAT 3. STAT proteins contain a Src Homology 2 (SH2) domain that mediates binding to the phosphotyrosine on the receptor complex. JAK phosphorylates STAT, causing it to dissociate from the receptor complex. The SH2 domain mediates binding to a second STAT protein causing dimerization [32]. The activated STAT complex translocate to the nucleus and function as a transcription factor (figure 7) [46].

Upon ligand binding the ERK/MAPK pathway is activated by recruitment of SHP-2 to a phosphorylated tyrosine residue on the gp130 receptor [reviewed in [48]]. JAKs phosphorylate and activate growth-factor-receptor-bound protein 2 (Grb-2), which is associated with Sos. Sos function as a GTP exchange factor (GEF) for Ras, causing activation of Ras and further signaling though ERK-MAPK (figure 7) [32, 51].

IL-6 binding also activates PI3K, which phosphorylates and thereby activate AKT [32, 52]. PI3K- Akt –NFκB pathway is part of the anti-apoptotic effect of IL-6 against transforming growth factor beta (TGF-β) signaling [52]. Inhibitor of Kappa B (IkB) inhibits NFκB, and is phosphorylated by a nemo complex upon activation. Phosphorylation leads to ubiquitination and degradation of IkB in the proteasome, causing NFκB to translocate to the nucleus and function as a transcription factor (figure 7) [32].

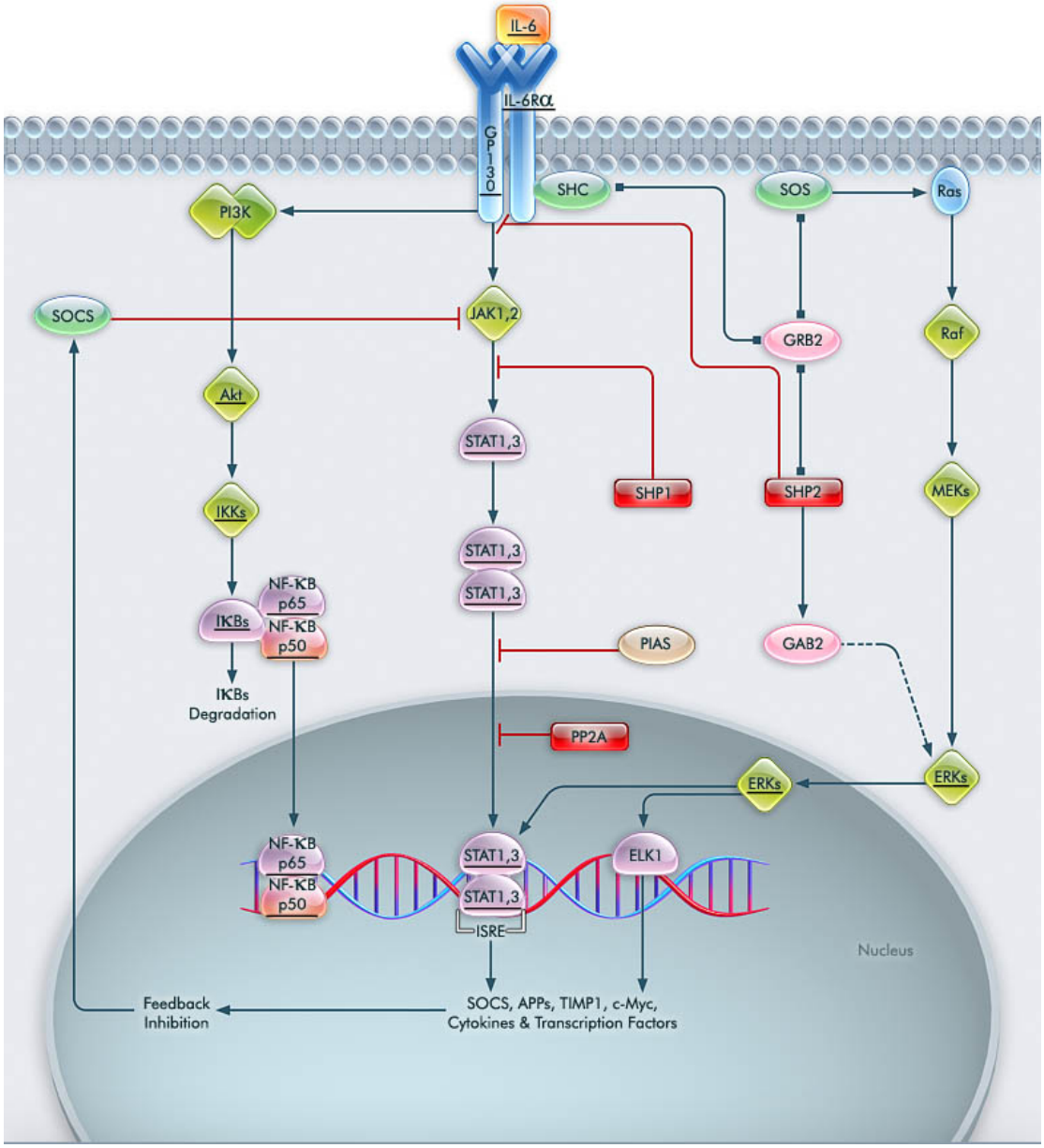

Figure 7 IL-6 pathway including JAK/STAT, ERK and PI3K/NFκB pathways[53].

2.5.2.1 IL-6 classic signaling and IL-6 trans-signaling

Only a few cell types such as macrophages, neutrophils, T-cells and hepatocytes express IL-6R on the cell surface, limiting the response of IL-6 [54]. On the contrary, gp130 is ubiquitously expressed. IL-6 can bind to a Soluble IL-6 Receptor (sIL-6R) and target cells that express gp130 [54]. IL-6 signals either though the classical signalling pathway, or through trans signalling (figure 8).

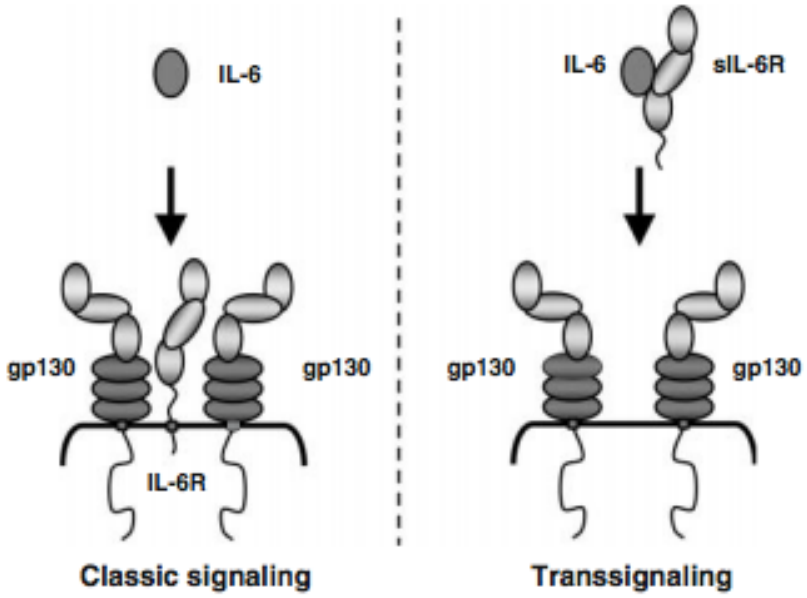

Figure 8 Classic and trans signalling of IL-6. In classic signalling IL-6 binds to the receptor complex on a cell surface. In cells that don't express IL-6R, IL-6 can dimerize with sIL-6R to induce signalling in cells expressing gp130 [44].

Recent studies suggest that IL-6 can be pro-inflammatory when trans-signalling and anti-inflammatory when signalling though membrane bound receptors [44, 55].

2.6 CRP

CRP is an acute-phase protein that is produced in the liver [56] and increases in response to tissue damage, inflammation or bacterial infection. It is widely used as a biomarker for infection and is measured by different laboratory techniques [57]. IL-6 is the main inducer of CRP, and the production can be enhanced in presence of IL-1 [58].

The human CRP molecule is composed of five nonglycosylated polypeptide subunits containing 206 amino acid residues each (figure 9) [reviewed in: [59]]

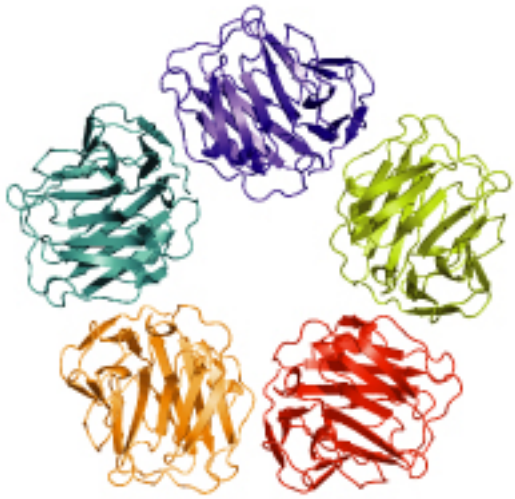

Figure 9 Three dimensional structure of the human C-reactive protein containing five nonglycosylated polypeptide subunits containing 206 amino acid residues each [60].

In healthy individuals, the concentration of CRP is <5mg/L. High plasma values can be observed 6-12 hours after an infection has occurred and can increase to a level above 200mg/L. Upon resolution of inflammation or tissue damage, CRP levels decline with the half-life being 15-25 hours [61]. A slightly elevated high sensitive-CRP (hs-CRP) concentration is considered to be a marker for chronic low grade inflammation, and is often detected in overweight and obese individuals [62].

2.7 ELISA

ELISA, Enzyme Linked Immunosorbent Assay, is an assay that uses antibodies and enzyme-mediated colour change to detect antigens. It can detect low concentrations of protein, peptides, hormones and other biological molecules [63]. A purified preparation of a known antibody and/or antigen is necessary to standardize the assay [39]. ELISA uses the basic concept of an antigen binding chemically to a specific antibody and a chromogenic substrate to induce a visible colour or fluorescence [39, 63]. The colour or fluorescence is measured to create a standard curve of known concentrations and calculate the concentrations of unknown samples. There are several variations to the method, it can be competitive, indirect or sandwich ELISA [63, 64]. In this thesis, sandwich ELISA was applied.

2.7.1 Sandwich ELISA

Sandwich ELISA is a modification of ELISA that is used to detect secreted products such as cytokines. Instead of binding the antigen directly to the plastic plate, an antigen-specific antibody is attached to the plate surface. The antigen binds with high affinity to the antibody, allowing detection of low concentrations in the sample [39].

A plate is coated with a known quantity of antibody and the antigen from a sample binds to the capture antibody [63]. Unbound antigen or other proteins are washed away with a wash solution [39] before a detection antibody is added to ”sandwich” the antigen. Lastly, a substrate is added which binds to the second antibody and is enzymatically converted to a detectable colour (figure 10). Sandwich ELISA is both specific and sensitive because it eliminates the need to purify the antigen from a mixture of other antigens [63].

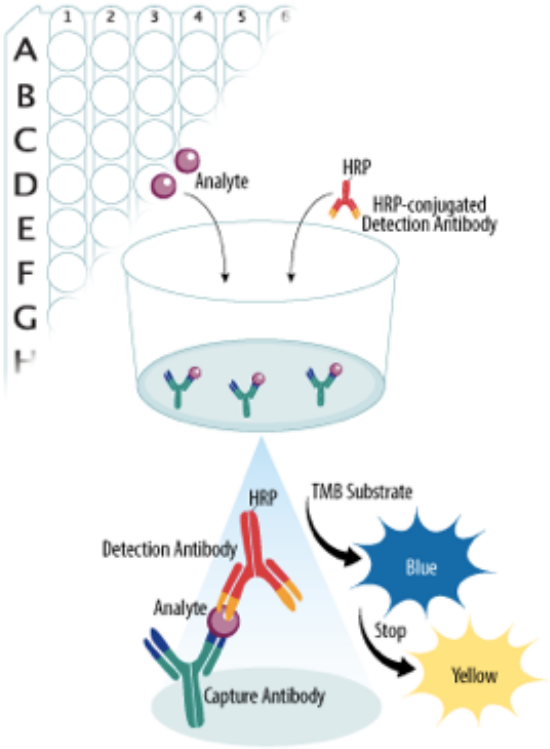

Figure 10 Sandwich ELISA. The wanted analyte is “sandwiched” between a capture antibody and detection antibody. Substrate, in this case Tetramethyl Benzidine (TMB), is added to enzymatically create a detectable colour [65].

2.7.1.1 HRP and TMB

Horseradish Peroxidase (HRP) is isolated from horseradish roots (*Armoracia rusticana*) and is one of the most widely used enzyme for immunodiagnostic products [66]. There are several peroxidase isoenzymes in the horseradish root, whereas Horseradish Peroxidase C (HRP C) is the most abundant. The protein is composed of 308 amino acid residues with one heme group localized between two domains, and two calcium atoms as showed in figure 11 [67]. The protein contains 8 neutral carbohydrate side chains attached to asparagine residues [66].

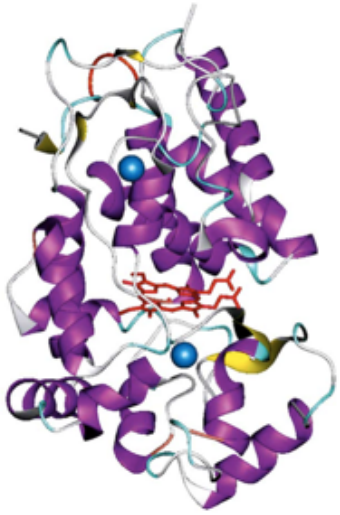

Figure 11 3D structure of HRP C. The heme group (coloured red) is located between two domains (distal and proximal domains), each containing one calcium atom (coloured blue) [67].

TMB is commonly used as a hydrogen donor for HRP, and requires the presence of H_2O_2. TMB is beneficial due to its high absorbance values and low background [66]. In presence of hydrogen peroxide, HRP catalyses oxidation of TMB and yield a blue product [68]. Addition of acidic solution yield a yellow colour in the solution [69].

2.7.1.2 Amplification of signal

Amplification of signal technologies is used to increase sensitivity of ELISA. High Sensitivity IL-6 ELISA kit from eBiosciense applies this technology from PerkinElmer [70].

Tyramide Signal Amplification (TSA) is a technology that amplifies the chromogenic signal used in ELISA. This technology results in higher sensitivity and allows detection of low abundant targets [70]. The amplification reagent is a phenolic compound that is activated by HRP, and is converted into a free radical intermediate that instantly bind to electron-rich regions of the target protein. The compound binds to predominantly tyrosine residues adjacently to sites where HRP is bound (figure 12) [70].

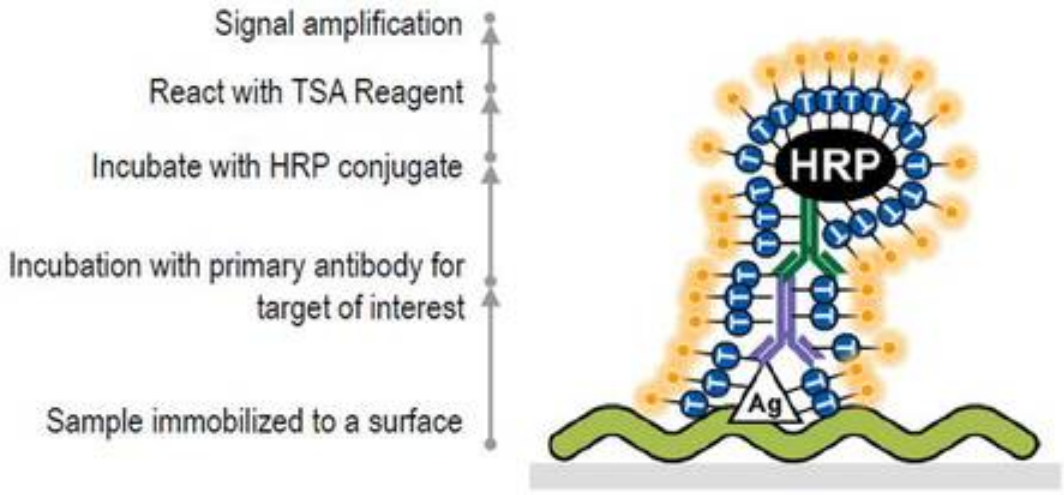

Figure 12 Antigen bound to a surface, antibody that binds to antigen (purple), HRP conjugate (green), TSA binding to tyrosine residues and amplification of signal [70].

2.8 Food and genome project at NTNU

The PLA_2 group at NTNU has conducted two studies to investigate how the calorie distribution of macronutrients in the diet affects chronic low-grade inflammation.

The pilot study, NTNU I, included five obese men with BMI>30 kg/m^2. Their daily diet were recorded for four days to map their macronutrient content. During the intervention, all subjects were instructed to eat four main meals and two in between meals with a ratio of 30 E% proteins, 40 E% fats and 30 E% carbohydrates. Fasting blood samples was collected at day 0 (baseline), 1, 2, 7, 14 and 28, which was the last day of the intervention. Biopsies of adipose tissue were collected at day 0,7 and 28. The diet was normocaloric, and immunoassays were performed on the samples of three men [71].

Gene expression time profiling was performed, and they found that several genes involving inflammation were down regulated as an effect of a moderate carbohydrate diet. The findings suggest that the composition of macronutrients affect low-grade inflammation and ultimately lifestyle diseases [71].

The main study, NTNU II, was a randomized, controlled, cross-over diet intervention trial. Thirty-two individuals finished the intervention. Two diets with different macronutrient composition were produced in powder form for the purpose of the study. AHC diet was based on the Norwegian and US dietary recommendations and composed of 65 E% carbohydrates, 15 E% proteins and 20 E% fats. The BMC diet was composed of 27 E% carbohydrates, 30 E% proteins and 43 E% fat. Each subject was given six of these meals each day. There were two study arms, either starting with AHC or BMC. In between was an eight-day wash-out period consisting of normal food [72]. Several changes were observed during the intervention which suggest that the BMC diet elicits a more anti-inflammatory response than the AHC diet [72].

This thesis is part of a third "food and genome" project, NTNU III. The aim is to further investigate how a balanced diet concerning the macronutrients affects low-grade inflammation. NTNU III is described in more detail in section 3.2.

2.9 Aims of the thesis

This thesis is a part of a study (NTNU III) executed by Ph.D. student Marian Førde and the PLA_2 group at NTNU. The aim of the study is to further investigate how a diet with a moderate load of carbohydrates affects low-grade inflammation in overweight women. There are several proteins and hormones of interest, both from the metabolic and inflammatory pathways. Inflammatory biomarkers such as cytokines (ex. IL-6 and TNF-α) and adipokines (ex. adiponectin and plasminogen Activator Inhibitor Type 1 (PAI-1)) will be of interest in this study [71]. In this master thesis the concentration of IL-6 was the main focus.

Hence, the aims for this thesis is to:

- Use ELISA to determine the concentration of IL-6 in blood serum of all the participants before the diets (baseline), after the AHC diet and after the BMC diet.
- Investigate if the concentration of IL-6 is altered during the BMC diet.
- Investigate if there is any correlation between IL-6 and CRP, and IL-6 and BMI.

3. Materials and methods

3.1 Reagents and material

All reagents and material used are listed below in table 1 and 2.

Table 1: Reagents and materials used for Human IL-6 High sensitivity ELISA kit from eBioscience.

Reagents, material	Supplier
Micro-well plate coated with monoclonal antibody to human IL-6	eBioscience
Biotin-Conjugate	eBioscience
Streptavidin-HRP	eBioscience
Human IL-6 standard	eBioscience
Control low	eBioscience
Control high	eBioscience
Assay buffer concentrate	eBioscience
Sample diluent	eBioscience
Amplification diluent concentrate	eBioscience
Amplification reagent I	eBioscience
Amplification reagent II	eBioscience
Wash buffer concentrate (PBS with 1% Tween)	eBioscience
Substrate solution (tetramethyl-benzidine)	eBioscience
Stop solution (1M phosoric acid)	eBioscience

Table 2: All reagents and materials used for Human IL-6 duoset ELISA kit from R&D systems.

Reagents, Material	Supplier
Capture antibody	R&D systems
Detection antibody	R&D systems
Recombinant standard	R&D systems
Streptavidin-HRP	R&D systems
Phosphate buffered saline (PBS)	OXID
Tween	Sigma Aldrich
Reagent diluent	R&D systems
Substrate solutions	R&D systems
Stop solution (0.5M H_2SO_4)	Self-prepared
Micro-well plates	Nunc

3.2 Subjects and replacement diet

All participating subjects were women with BMI>30. All subjects had to undergo a through health check before the intervention. Exclusion criteria was no inflammatory disease, no medication, and otherwise healthy.

The intervention lasted for 13 days. The first day a blood sample was collected, a weight check was done, and they were given 3 days of AHC diet. The fourth day a blood sample was collected, weight was controlled, and they were given 10 days of BMC diet. After 14 days a final blood sample was collected and weight was controlled (figure 13). All blood samples were collected in the morning after an overnight fast.

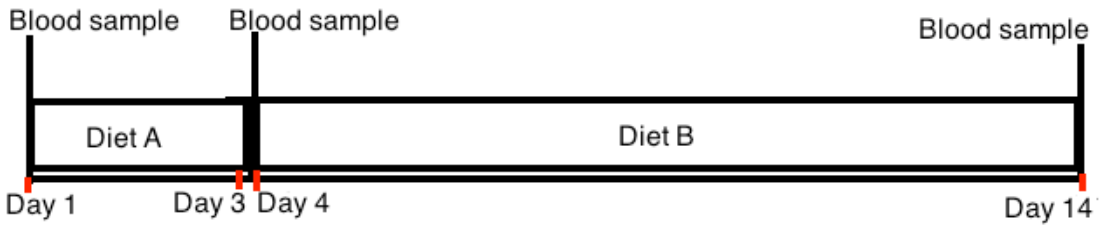

Figure 13 Schematic overview of the intervention. The subjects were given three days of diet A (AHC) and 10 days of diet B (BMC). Fasting blood samples were drawn before the intervention, between the diets and after BMC diet.

AHC is a high carbohydrate diet with a calorie distribution of 65 E% carbohydrates, 15 E% proteins and 20 E% fat. BMC is a balanced diet with regards to macronutrients, composed of 27 E% carbohydrates, 30 E% proteins and 43 E% fat. Nutrient content of the diets is summarized in figure 14.

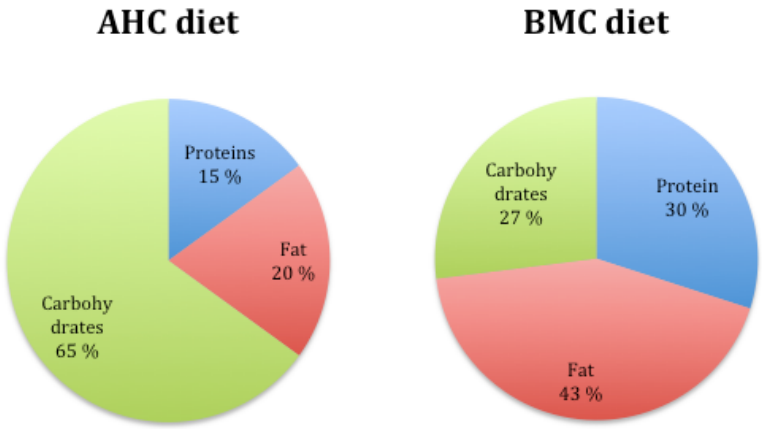

Figure 14 Calorie distribution between macronutrients in E% for AHC and BMC diet.

Both diets were normocaloric and the subjects were instructed to keep their activity level as usual. The concentration of IL-6 and other pro-inflammatory cytokines is elevated in obese individuals [28], and is shown to decrease following weight loss [43]. The main focus of this study is to investigate the effect of diet change, not weight loss.

3.3 Blood processing and handling

Blood samples were collected at "Forskningsposten" at St.Olavs Hospital, Trondheim. All the samples were taken in the morning after an overnight fast. The samples were left to coagulate in room temperature for 30 minutes, before centrifugation at room temperature for 10 min at 1300 relative centrifugal force (RCF). The samples were aliquoted and frozen at -20°C for approximately 2 weeks before they were moved to a -80°C freezer for long time storage.

Blood serum is the liquid part of the blood containing proteins and other biological molecules. Cells and clotting factors are given time to clot so it can be separated from the liquid serum (figure 15). If the blood is left for less than 30 minutes it is likely that there are still cellular components in the serum. However, if the sample is left for too long it is likely that the cells will lyse and leak cellular components in to the serum [73].

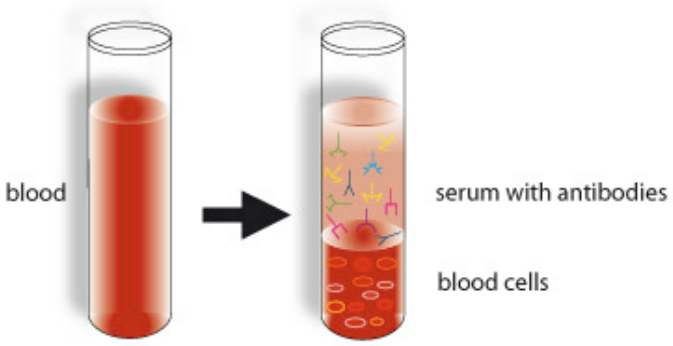

Figure 15 Blood is given time to clot so it separates from the liquid serum containing biological molecules such as antibodies [1].

Temperature is an important factor when handling blood samples. Protein and enzyme activity is affected by temperature, so long term storage of samples should be at -80°C [73]. The level of IL-6 remain stable under freeze and thaw cycles in blood serum [74].

3.4 ELISA kits from eBioscience and R&D systems

IL-6 concentrations in blood serum were found using a high sensitivity ELISA kit from eBioscience and Human IL-6 Duoset kit from R&D systems. All samples were analysed using the Human IL-6 Duoset kit from R&D with a range of 9.38-600 pg/ml. Samples not detected within this range were detected using High sensitivity ELISA kit from eBioscience.

3.4.1 R&D systems human IL-6 Duoset

Human IL-6 ELISA kit from R&D systems provides a range of 9.38-600 pg/ml [75]. The protocol from R&D systems was followed with minor adjustments [75].

PBS solution was prepared by dissolving ten PBS tablets in 1 litre of distilled water. Wash buffer used was made of PBS with 0.05% Tween.

Capture antibody was diluted in PBS and a 96 micro-well plate was immediately coated. The plate was incubated over night at 4°C. After the coating period the micro-well plate was emptied and washed five times with 300µl wash buffer. 300µl reagent diluent was added to each well to block the coating reaction with incubation for one hour at 25°C. Five washing steps were performed before standards, blanks and samples were added in duplicates to the micro-wells. The plate was incubated overnight at 4°C. After five wash steps, detection antibody was added and incubated on a microplate shaker for 2 hours at 400 Rotations Per Minute (rpm). The micro-wells were washed to remove excess detection antibody and to avoid unspecific binding. 100µl streptavidin-HRP was added to each well and incubated for 20 min at 400rpm. The micro-wells were washed to remove excess streptavidin-HRP, after witch 100µl substrate was applied in all wells. The plate was incubated in the dark for 20 min at 400rpm. Following incubation stop solution was added and absorbance was immediately read at 450 nm, with a reference wavelength 540nm using a FLUQstar-Omega plate reader.

3.4.2 eBioscience high sensitivity ELISA

High sensitive ELISA kit from eBioscience provides a range of 0.08-5.00 pg/ml with a sensitivity of 0.03 pg/ml [74]. The protocol provided from eBioscience was followed [74].

Precoated plates (coated with anti-human IL-6) were washed twice with 400µl wash buffer before applying standards, controls and samples in duplicates. 50µl diluted biotin conjugated anti-human IL-6 was added to each well before incubation at 400rpm for two hours. After incubation the plate was washed six times to remove unbound biotin conjugate and prevent unspecific binding. 100µl streptavidin-HPR was added to each well to bind biotin conjugated IL-6 antibody. The micro-well plate was then incubated for one hour at 400rpm. A wash step was performed and100µl amplification solution (biotinyl-tyramide) was applied before incubating for 15 minutes at 400rpm. The plate was washed six times to remove any unbound amplification solution. After the wash step, 100µl amplification solution II (streptavidin-HRP) was added to each well before incubating at 400 rpm for 30 minutes. Excess amplification solution II was removed by wash and TMB substrate solution with HRP was applied in all wells. The plate was incubated in the dark for about 20 minutes until the A1 standard turned dark blue. The reaction was terminated by adding stop solution (acid). The absorbance was measured at 450nm with reference wavelength at 620nm using a FLUQstar-Omega plate reader.

3.5 CRP

High-sensitivity CRP was measured at the department of medical biochemistry at St.Olavs hospital in Trondheim using the instrument Roche Modular P [76].

CRP from serum or plasma sample binds to specific antibodies that are connected to latex particles. Binding of CRP causes agglutination of the particles and the solution becomes turbid. Absorbance is measured at 546 nm, and is proportional to the CRP-concentration [76].

3.6 Statistical analysis

Standard curves were created by doing a four-parameter logistic curve fit using ELISA software at myassays.com. Further analysis was done in SPSS statistics version 21. Differences was considered significant when $p<0.05$.

A Kolmogorv-Smirnov test was done to check for normality. The Friedman test was applied to find significant differences between groups. For pairwise comparison, a Wilcoxon signet rank test was applied with a Bonferroni adjusted alpha value.

The Spearman Rank Correlation Test was used to investigate correlation between IL-6 and CRP and IL-6 and BMI. The spearman rank correlation test is non-parametric and used to find relations between two variables. The Spearman Rank Correlation Coefficient (r) shows high level of correlation when close to 1, either positive or negative [77].

4. Results

4.1 IL-6 concentration detected with ELISA

IL-6 is commonly used as a marker of inflammation. The aim for this thesis is to determine IL-6 concentration in blood serum for all participating subjects. IL-6 concentrations at baseline, after AHC and after BMC were detected using sandwich ELISA. Two different ELISA kits were used. The samples were analysed using human IL-6 ELISA kit from R&D with a range of 9.38-600 pg/ml. Samples not detected within this range were analysed using a high sensitive ELISA kit from eBioscience with a range of 0.08-5.00 pg/ml.

4.1.1 Standard curves

To calculate the concentrations of unknown samples using ELISA, a standard curve must be prepared for each 96-well plate. Each 96-well plate had its own standards with 7 samples of known concentrations. Standard curves were created performing a four-parameter logistic curve fit at myassays.com. One standard curve from each kit is presented in figure 16 and 17.

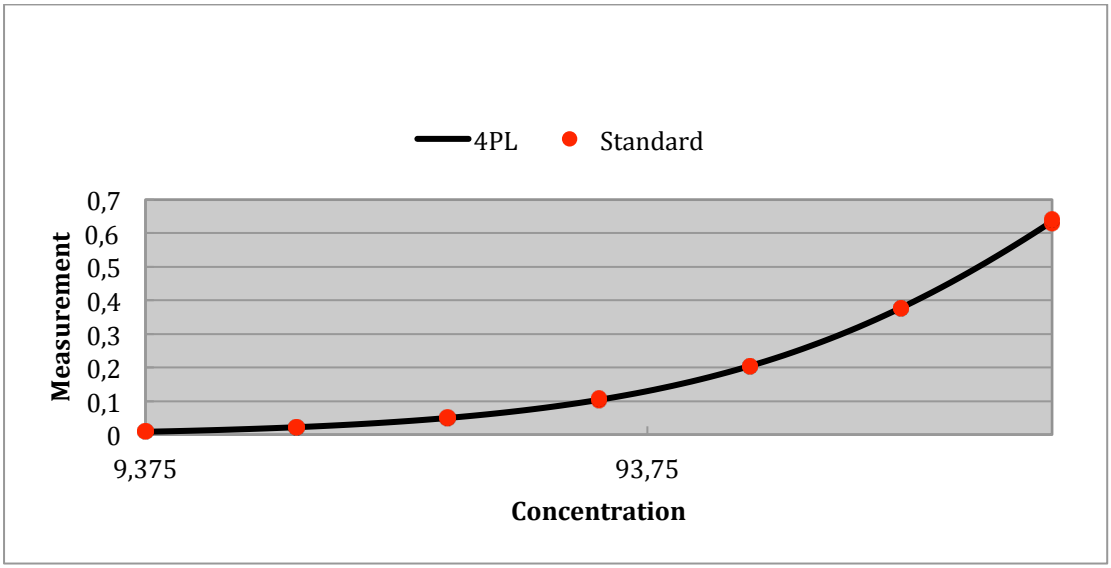

Figure 16 Standard curve for Human IL-6 Duoset kit from R&D systems created with a four-parameter logistic curve at myassay.com. 7 samples of known concentrations (red) used to create the standard curve.

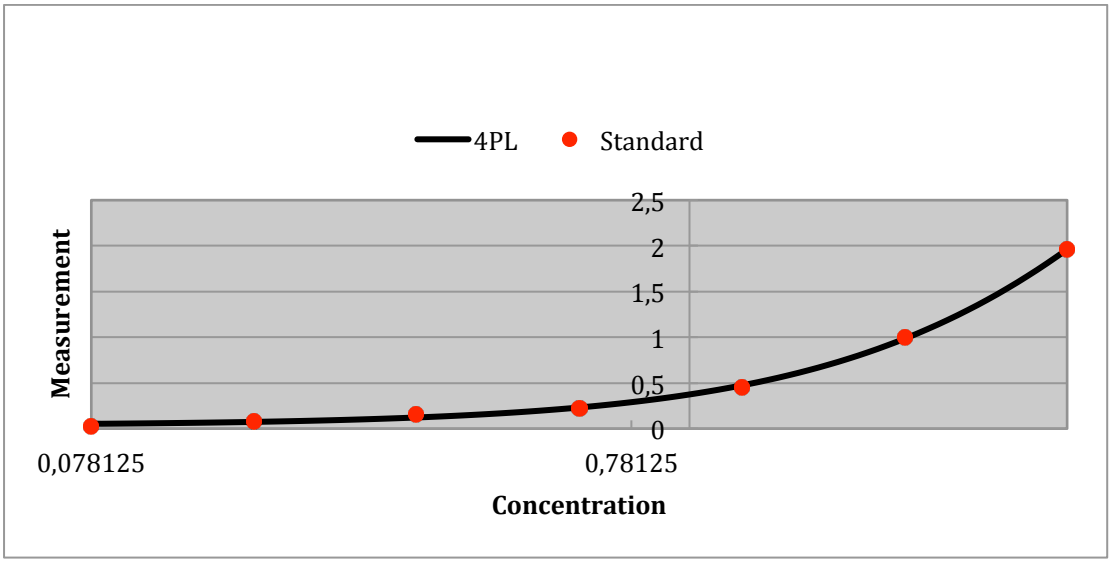

Figure 17 Standard curve for Human IL-6 High sensitivity ELISA kit from eBioscience created with a four-parameter logistic curve at myassay.com. 7 samples of known concentrations (red) used to create the standard curve.

4.1.2 IL-6 concentrations for all subjects

All three samples; baseline, AHC and BMC from all 28 participating subjects were first analysed using human IL-6 ELISA kit from R&D systems. IL-6 concentration for subject A3, A22, A38, A39, A44 and A48 was detected within the range of this kit. All Samples not detected on a human IL-6 ELISA kit from R&D systems were analysed using a high sensitivity ELISA kit from eBioscience. IL-6 concentrations from the restoring 22 subjects were detected within the range of this kit.

IL-6 concentrations detected on the human IL-6 ELISA kit from R&D❖ had a range of 15.55-13778.2 pg/ml. IL-6 concentrations detected on the high sensitive ELISA kit from eBioscience had a range of 0.1853-4.196 pg/ml. A22 was excluded as an obvious outlier and is not part of further statistical testing. IL-6 concentrations (pg/ml) for all subjects at baseline, AHC and BMC are presented in table 3.

Table 3: IL-6 concentrations for all subjects at baseline, AHC and BMC. Samples were analysed using human IL-6 ELISA kit from R&D❖ and high sensitive ELISA kit from eBioscience. A22 is excluded as an obvious outlier for further analysis.

	IL-6 (pg/ml)		
Subject	**Baseline**	**AHC diet**	**BMC diet**
A1	2.313	1.701	2.192
A3❖	472.2	353	352.5
A4	0.2517	1.069	0.1853
A8	3.381	3.002	3.091
A22	10026.3	10970	13778.2
A23	2.193	1.968	1.34
A24	0.5477	0.2433	0.6609
A26	1.051	1.315	1.705
A27	1.677	3.637	4.196
A28	3.937	1.43	0.7835
A29	0.8824	1.557	2.462
A30	1.409	1.079	1.398
A31	0.6171	0.6054	0.6689
A32	0.8815	0.8896	0.8844
A33	1.379	1.331	0.4025
A35	1.537	1.867	2.132
A38❖	20.42	19.05	24.17
A39❖	12.55	17.01	24.51
A40	0.8588	0.8257	0.9587
A41	0.6827	0.8857	0.9511
A42	1.242	0.9175	0.6057
A43	0.6092	2.064	2.325
A44❖	87.15	91.84	101.7
A45	1.228	2.551	3.011
A46	1.148	1.269	1.379
A47	1.182	0.875	0.9015
A48❖	124.2	116	182.7
A49	0.4877	0.3676	0.3834

To summarize, IL-6 concentration for 6 subjects were detected on the human IL-6 ELISA kit from R&D. IL-6 concentrations from the restoring 22 subjects were detected on a high sensitive ELISA kit from eBioscience. One subject was recognized as an obvious outlier and is excluded from further analysis.

4.2 Alteration of IL-6 concentration

The aim of this thesis was to investigate if the alteration of dietary macronutrients affected IL-6 concentration in blood serum from 28 obese females. The Kolmogorv-Smirnov test showed no normality in the data ($p<0.05$). All further testing was done in a non-parametric manner.

To visualise differences of IL-6 concentration between the three sampling points, median ΔAHC-baseline, and median ΔBMC-AHC were calculated using excel. The chart in figure 18 indicates a small decrease in IL-6 concentration (0.033 pg/ml) from baseline to AHC and a small increase of concentration (0.133 pg/ml) from AHC to BMC.

A Friedman test was applied to compare the concentrations detected at baseline, AHC and BMC and evaluate if any of the observed alterations in figure 18 can be considered significant. No significant differences were detected ($p = 0.064$). The p-value was close to significant, suggesting a trend in IL-6 alteration.

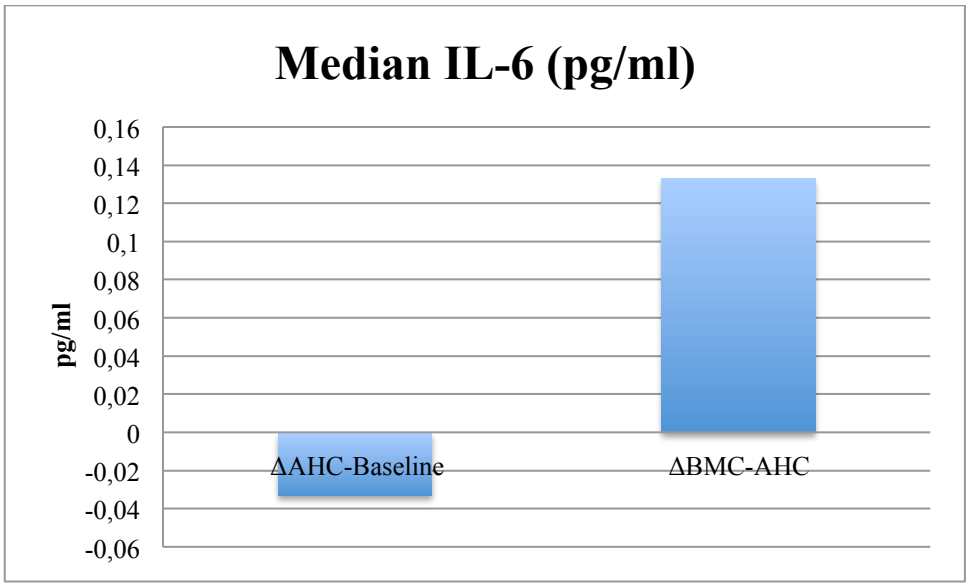

Figure 18 The concentration of IL-6 in 28 obese females at three different time points were found using ELISA kits from R&D and eBioscience. The median IL-6 concentration (pg/ml) for ΔAHC-baseline, and ΔBMC-AHC (pg/ml) were calculated to evaluate any variations. The Friedman test showed no significant differences between IL-6 concentrations detected at baseline, AHC and BMC, $p>0.05$.

No significant differences were detected between the three sampling points. However, the p-value was close to significant (p = 0.067) so a pairwise comparison was done to evaluate differences between the three sampling points.

A Wilcoxon Signet Rank Test was applied with a Bonferroni adjusted alpha value (α = 0.017) to look for pairwise differences. As seen in table 4, there was no significant change between baseline-AHC (p = 0.876), baseline-BMC (p = 0.230) or AHC-BMC (p = 0.064).

Table 4: P-values for pairwise comparison between baseline-AHC, baseline-BMC and AHC-BMC using a Wilcoxon Signed Rank Test with a Bonferroni adjusted alpha value (α = 0.017). No significant change was detected, p>0.017.

	P-value
Baseline-ACH	0.876
Baseline-BMC	0.230
AHC-BMC	0.064

To summarize, no significant differences of IL-6 concentrations were found between baseline, AHC and BMC.

4.3 Correlation

Studies show that IL-6 correlates with both CRP and BMI [29]. To investigate these relations, a Spearman rank correlation test was done to look for correlation between the IL-6 concentrations, BMI and CRP levels in a non-parametric manner.

4.3.1 IL-6 and BMI

A Spearman Rank Correlation test was done to investigate the relationship between IL-6 concentration and BMI at the three sampling points: baseline, AHC and BMC. BMI for all subjects is listed in appendix C. The Spearman rank correlation coefficient (r) and shared variance (%) is calculated for all three points. As seen in table 5, no significant correlation was found (p>0.05). Correlation at baseline and AHC is small, r<0.250. A trend can be observed at BMC, where IL-6 and BMI has a medium correlation with r = 0.340 and shared variance of 11.56% (p = 0,089).

Table 5: Spearman rank correlation coefficient (r) estimates correlation between IL-6 concentrations and BMI at baseline, after AHC diet and after BMC diet. Shared variance is calculated for all three sampling times. No significant correlation between IL-6 and BMI was found, p>0.05. A trend can be observed at BMC, where IL-6 and BMI has a medium correlation (r = 0,340, p = 0,089).

Correlation between:			r	Shared variance (%)	P-value
IL-6 baseline	**-**	**BMI baseline**	0.076	0.58	0.712
IL-6 AHC	**-**	**BMI AHC**	0.244	5.95	0.244
IL-6 BMC	**-**	**BMI BMC**	0.340	11.56	0.089

4.3.2 IL-6 and CRP

A Spearman rank correlation test was done to investigate the relationship between IL-6 concentration and CRP at baseline, AHC and BMC. CRP measured for all subjects at the three sampling points is listed in appendix D. The Spearman rank correlation coefficient (r) estimates correlation, and shared variance (%) is calculated. As seen in table 6, no significant correlation was found (p>0.05). Correlation at baseline and AHC is almost zero, and a small correlation at BMC can be observed with a shared variance at 4.62%.

Table 6: Spearman rank correlation coefficient (r) estimates correlation between IL-6 concentrations and CRP at baseline, after AHC diet and after BMC diet. Shared variance is calculated for all three sampling points. No significant correlation between IL-6 and CRP was found, p>0.05.

Correlation between:			r	Shared variance (%)	P-value
IL-6 baseline	**-**	**CRP baseline**	-0.002	0.04	0.993
IL-6 AHC	**-**	**CRP AHC**	0.155	2.40	0.450
IL-6 BMC	**-**	**CRP BMC**	0.215	4.62	0.291

5. Discussion

5.1 ELISA and technical variation

The concentration of IL-6 in human blood serum is normally <3-4 pg/ml [43]. In obese individuals the concentration can be slightly elevated [28]. Two different ELISA kits were used to determine IL-6 concentration, as few ELISA kits have a range that covers both the lowest and highest concentrations found. All samples were analysed using a Human IL-6 ELISA kit from R&D with a range of 9.38-600 pg/ml. Samples not detected within this range were analysed using a high sensitive ELISA kit from eBioscience with a range of 0.08-5.00 pg/ml. It is beneficial to test all samples on one kit to avoid any variance occurring between different batches and brands. This was not possible due to large variation in the IL-6 concentration between individuals. All three samples from the same subject was run on the same micro-well plate, so no inter-plate variations would affect the alteration of IL-6 levels within one subject. All plates have separate standard curves, from which the results were calculated. Standards were used as a control to detect and avoid inter assay variance. Two standard curves are presented in section 4.1.1, one example from R&D kit, and one from the eBiosience (figure 16 and 17).

All samples were tested in duplicates to reduce technical variance. Standard deviation between duplicates should preferably be under 5%, and below 10% was accepted (appendix A). All subjects have IL-6 concentration within the same range between the three sampling points (appendix B).

Protocols for blood sampling and handling was followed carefully to reduce any analytical variations that is not related to the inflammatory status.

5.2 Statistical methods and sample size

The power of a test is the probability of rejecting the null hypothesis when it is false. Statistical power is positively correlated with sample size, when the sample size is large, power is not an issue and even small relations can be detected. In a somewhat smaller sample size, small relations can be more difficult to detect [78].

28 women finished the diet intervention. One obvious outlier was excluded from the results, leaving the final sample size to 27. The method of Jørstad et.al. [79] was used

to calculate sample size in NTNU III based on previous studies. It was estimated that a sample size of 19 would be sufficient to obtain satisfactory statistical power (0.80) when investigating gene expression in response to a diet. Proteins and hormones vary differently in response to dietary alteration than gene expression, hence separate power tests need to be calculated to obtain a sufficient sample size. There was not enough background knowledge to perform a separate power-test for IL-6 and other proteins of interest before the intervention. A study done to investigate the effect of a high fish diet on secreted cytokines estimated that a sample size of 40 subjects would be sufficient to detect a CRP alteration of 13% [80]. The IL-6 concentrations detected in this thesis vary to a bigger extent, indicating that a sample size beyond 40 is required to obtain sufficient statistical power.

The Kolmogorv-Smirnov test showed no normality in the data so further statistical testing was done in a non-parametric manner. The Friedman test is non-parametric, and is used when samples from same participants under three or more different conditions is investigated. The test ranks the values according to each other and calculate mean rank to determine statistical significance [77]. When the data is not normally distributed the mean and median values can give a faulty impression of alteration in IL-6 concentration. To simply investigate at the differences in IL-6 concentration, median ΔAHC-baseline and ΔBMC-AHC were calculated and presented in figure 18. Delta values give a better representation of the alteration in concentrations, which is evaluated by the Friedman test. The Friedman test compared the raw IL-6 concentrations detected at baseline, AHC and BMC. No significant differences were found ($p = 0.067$).

Since the p-value was close to significant, a post hoc test was done to investigate trends between baseline-AHC, baseline-BMC and AHC-BMC. The Wilcoxon signed rank test, a non parametric test for pairwise comparison [77], was applied with a Bonferroni adjusted alpha value. Three tests were conducted and the α-value was adjusted from $\alpha = 0.05$ to $\alpha = 0.017$. Post hoc tests showed no significant change in IL-6 concentration between baseline-AHC ($p = 0.876$), baseline-BMC ($p = 0.230$) or AHC-BMC ($p = 0.064$).

In addition, we investigated whether the delta values calculated showed any significant differences compared to the raw IL-6 concentrations. The delta values

show more normality than the raw data, but not significantly. A pairwise t-test between Δbaseline-AHC and ΔAHC-BMC was done to compare those results with those from the non-parametric tests. The pairwise t-test showed no significant differences in IL-6 concentration.

5.3 IL-6 concentration

The concentration of IL-6 detected had a range of 0.1853-13778.2 pg/ml. One obvious outlier was excluded leaving a range of 0.1853-472.2 pg/ml. Delta values calculated indicates a small reduction of IL-6 concentration after AHC diet (-0.033 pg/ml), and a small increase after the BMC diet (0.133 pg/ml). The Friedman test and Wilcoxon signed rank test showed no significant differences in IL-6 concentration after the diets.

Due to the small p-value, it appears like there is a trend with an increase of IL-6 concentration from AHC to BMC. During the post hoc testing the alpha value was adjusted to reduce the chance of obtaining type 1 errors. With the Bonferroni adjusted alpha value, the p-value is not close to significant. Hence, we can conclude that the alteration of dietary macronutrients did not cause a significant change of IL-6 in the participating subjects. This can be visualised by the graphs in appendix B, showing IL-6 concentration for all individuals at all three sampling points. No obvious trend or change can be observed.

The findings in this work differs from the findings in NTNU II, where IL-6 was significantly reduced in response to the BMC diet [72]. There are several differences between these two studies that should be acknowledged. In NTNU III there are only female subjects, and the mean BMI is higher. The nature of the intervention is also different. NTNU II contained a washout period between the two diets, on contrary, subjects started on the BMC diet immediately after AHC during NTNU III. These are all factors that could contribute in explaining the different results.

Numerous studies have been done to investigate the effect of weight loss and different macronutrient composition on the inflammatory status of the body. The anti-inflammatory effect of weight loss has been established [30], whereas the effect of dietary change is more difficult to verify. A low carbohydrate diet has been shown to elicit a positive effect on the inflammatory status in patients with T2D. Low fat diets

can induce the same amount of weight loss, without the positive effects on inflammation [81]. Diets very low on carbohydrates (>12 E%) also decrease the amount of pro-inflammatory cytokines compared to a low fat diet [82]. A study showed that weight reduction from a low carbohydrate and a low fat diet did not correlate with the amount of secreted cytokines. This indicates that the anti-inflammatory effect is induced by the macronutrients and not the weight loss alone [82]. Both the amount and quality of dietary carbohydrates can have an effect on chronic low-grade inflammation. Low GI food can reduce hs-CRP with a normocaloric diet containing the same amount of carbohydrate, only differing in GI. A low GI diet does not have the same effect on IL-6, indicating that CRP could be more sensitive to dietary change than IL-6 [83].

The results obtained in this thesis show no alteration in IL-6 concentration after a balanced carbohydrate diet. Still, it remains to see if the total inflammatory profile is improved. Several of the studies mentioned above reduced the amount of carbohydrates to less than 40 E%. It is possible that the BMC diet is not extreme enough to lower the concentration of IL-6 in highly obese woman during a 10-day intervention. Different studies recruit participants with different BMI criteria. In all the above mentioned studies which indicated that a low or moderate carbohydrate diet down regulated inflammatory markers, participating subjects had a BMI>25 [72, 81, 82]. On the contrary, all subjects in NTNU III had a BMI>30. Studies that found a significant decrease in inflammatory markers following a low carbohydrate diet had a sample size>40 [81, 82], while NTNU III had 27 participants. Both BMI, sample size and intervention time could affect the alteration of inflammatory markers such as IL-6. However, recent research indicates that the composition of macronutrients in the diet affects inflammation, and that a low carbohydrate diet may be metabolically beneficial. Further research is needed to investigate the optimal macronutrient composition.

5.3.1 Correlation with BMI and CRP

IL-6 concentration has been shown to correlate with both CRP concentration and BMI [29]. The Spearman Rank Correlation Coefficient showed no correlation between IL-6 and CRP, and IL-6 and BMI in our data. The sample size in this study is quite small, and small relations and correlations can be difficult to detect.

Previous studies have shown significant correlation between IL-6 and BMI in obese subjects [29, 81], whereas the change in IL-6 after a diet intervention is shown not correlate with the change in BMI [81]. We could therefor expect a correlation between IL-6 and BMI at baseline, but neither after AHC nor BMC. No significant correlation was observed at the three sampling points, although there was a medium correlation at BMC ($p = 0.089$). The lack of correlation at baseline is most likely due to the small sample size.

5.4 IL-6 as an inflammatory marker

No significant differences of IL-6 concentration were found between baseline, AHC and BMC. As stated earlier, this differs from previous studies. It is however important to remember that IL-6 is not only part of obesity linked low-grade inflammation. IL-6 is part of both the acute phase response [21], bone homeostasis and metabolism [44].

IL-6 is only one of the proteins of interest in NTNU III. To draw a full conclusion it is necessary to look at the inflammatory profile as a whole, and not just one cytokine. We can however see a trend or indication by investigating IL-6.

5.5 Metabolically healthy but obese

Some obese individuals appear to be resistant to the metabolic abnormalities associated with obesity. The literature describes them as Metabolically Healthy but Obese (MHO) [84, 85]. There is evidence that about 25% of the obese population meet the criteria for MHO [85]. MHO individuals have high insulin sensitivity, low ectopic fat and low inflammation among other factors that separates them from metabolically abnormal obese (figure 19) [reviewed in [86]].

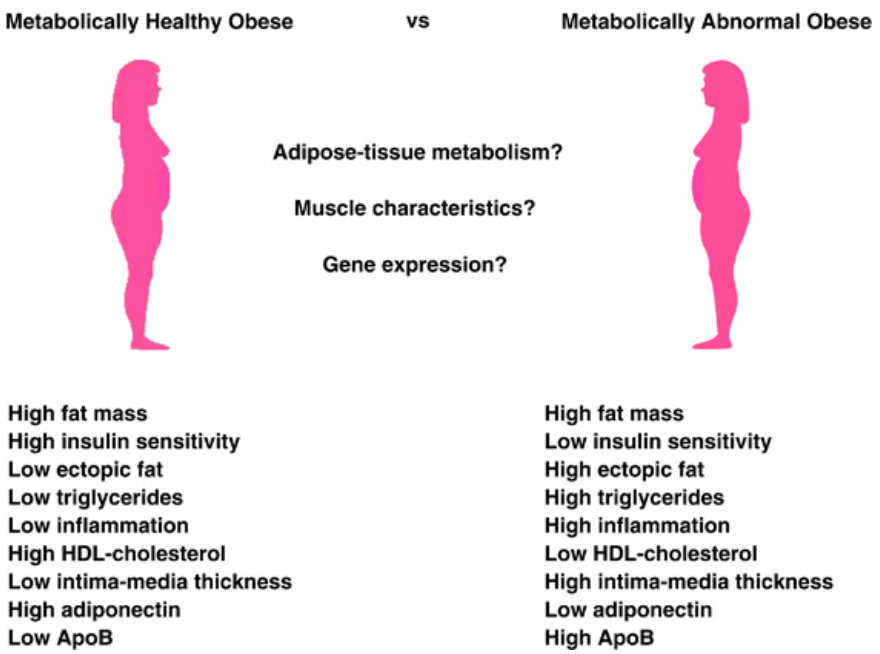

Figure 19 Factors separating metabolically healthy from metabolically abnormal obese [86].

Knowing that about 25% of obese individuals are metabolically healthy, it may be necessary to further increase the sample size to detect variances in metabolically abnormal obese. Other than using IL-6 as an inflammatory marker, the research done in this thesis cannot determine whether or not any MHO individuals participated in this study. It could however be detected further into NTNU III.

5.6 Limitations

Some limitations need to be acknowledged in this study. What is already mentioned is the sample size. A greater sample size would make it easier to detect small alterations between groups, and increase the power of the statistical tests. Since there was no normal distribution in this data, non-parametric tests had to be used and the power is further decreased.

Subjects were followed during the intervention. They were given the meal replacements and recommendations on how to distribute the meals throughout the day. Still, working with humans one can never have complete control of neither intake nor activity.

5.7 Further research

Since the results of this thesis show no significant change of IL-6, it will be interesting to look at the whole inflammatory profile and all the proteins of interest in this study. As stated, this thesis is part of NTNU III, so further work is already in progress. With knowledge about cytokines, insulin, glucose, leucocytes and other biological molecules it will be easier to see if there is a down regulation of inflammatory mediators after a change in dietary macronutrients.

In a longer term, it would be interesting to do studies with a larger sample size to increase the power of the statistical tests. Larger studies would also be beneficial for finding the best markers for chronic low-grade inflammation, making it easier to detect in the future. It would also be interesting to do studies with both males and females of different age and ethnicity to see how these variables affect how dietary macronutrients alter the inflammatory profile.

Intervention time could also be prolonged in future studies to investigate how the inflammatory profile changes over a longer period of time with a balanced macronutrient diet.

A better understanding of how dietary macronutrient composition affect inflammation can have a large impact on general health worldwide. If people can eat better to reduce chronic low-grade inflammation, the total risk for CVD, T2D and other life style diseases may be drastically reduced.

6. Conclusion

IL-6 concentrations in blood serum from 28 subjects with BMI>30 were detected using sandwich ELISA. Samples were analysed at baseline, after AHC diet and after BMC diet. No significant alteration of IL-6 was detected. No significant correlations were found between IL-6 and CRP or IL-6 and BMI. A trend can be observed with a medium correlation between BMI and IL-6 after BMC (P = 0,089).

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Appendix

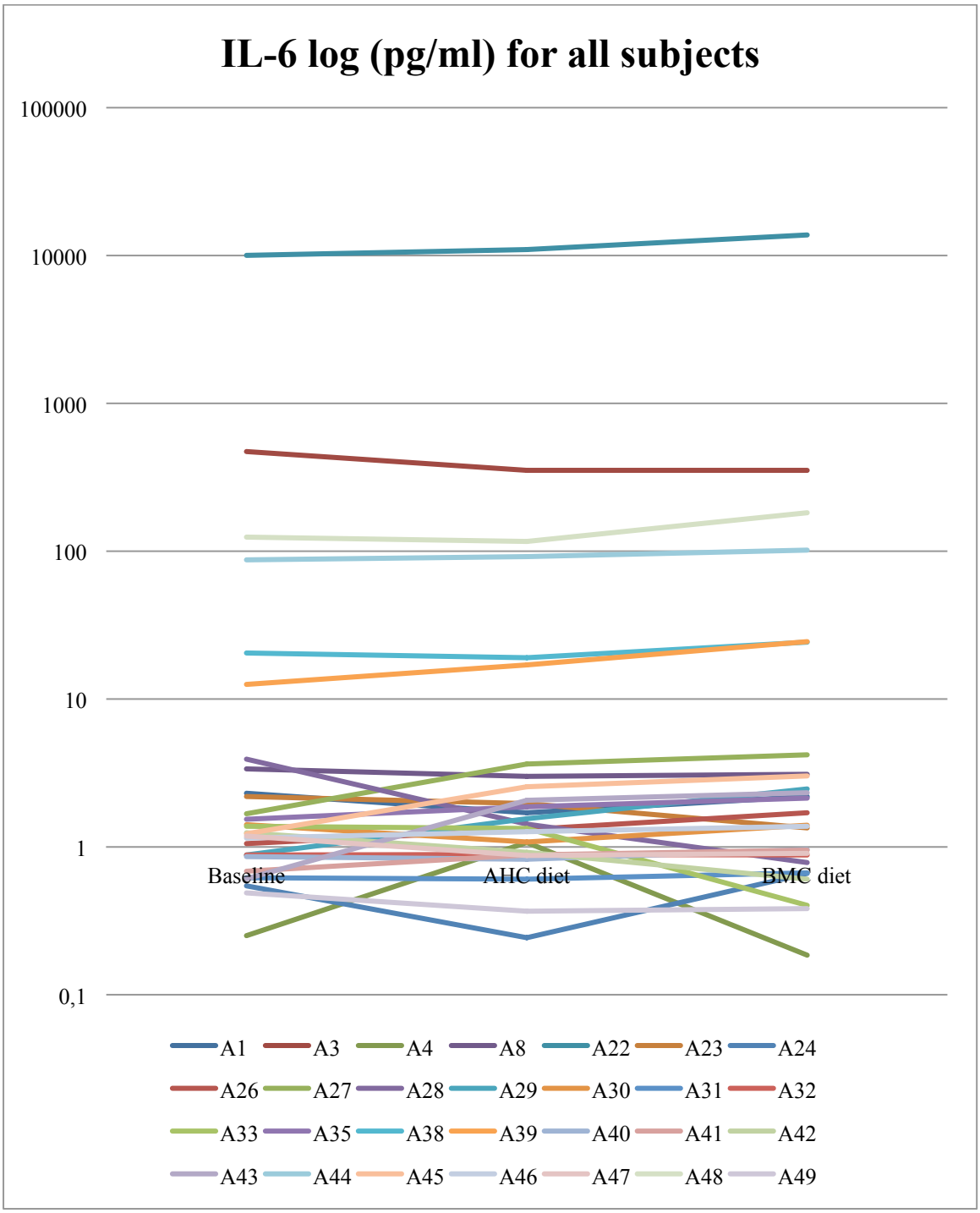
A: Raw data IL-6

Table 1: Raw data (OD) in duplicates for all individuals at baseline, after AHC diet and after BMC diet. Standard deviation (std.) is calculated for all duplicates.

	OD baseline			OD AHC			OD BMC diet		
Subject	**1**	**2**	**std.**	**1**	**2**	**std.**	**1**	**2**	**std.**
A1	0.551	0.606	3 %	0.459	0.369	4 %	0.586	0.504	4 %
A3	0.536	0.536	0 %	0.434	0.431	0 %	0.426	0.438	1 %
A4	0.188	0.183	0 %	0.327	0.314	1 %	0.171	0.158	1 %
A8	0.737	0.78	2 %	0.667	0.696	1 %	0.702	0.697	0 %
A22	0.228	0.024	10 %	1.431	1.487	0 %	0.319	0.317	0 %
A23	0.575	0.515	3 %	0.513	0.454	3 %	0.367	0.285	4 %
A24	0.173	0.122	3 %	0.138	0.091	2 %	0.19	0.19	0 %
A26	0.289	0.316	1 %	0.337	0.364	1 %	0.257	0.278	1 %
A27	0.407	0.407	0 %	1.02	0.895	6 %	1.12	1.12	0 %
A28	1.1	0.992	5 %	0.355	0.337	1 %	0.206	0.215	0 %
A29	0.237	0.22	1 %	0.37	0.384	1 %	0.608	0.633	1 %
A30	0.343	0.393	3 %	0.293	0.322	1 %	0.231	0.252	1 %
A31	0.239	0.217	1 %	0.229	0.223	0 %	0.247	0.226	1 %
A32	0.274	0.271	0 %	0.287	0.261	1 %	0.275	0.271	0 %
A33	0.415	0.311	5 %	0.379	0.328	3 %	0.164	0.167	0 %
A35	0.371	0.373	0 %	0.454	0.459	0 %	0.533	0.523	1 %
A38	0.03	0.034	0 %	0.026	0.034	0 %	0.037	0.038	0 %
A39	0.025	0.016	0 %	0.025	0.029	0 %	0.037	0.039	0 %
A40	0.225	0.223	0 %	0.223	0.213	1 %	0.246	0.24	0 %
A41	0.192	0.195	0 %	0.232	0.226	0 %	0.242	0.241	0 %
A42	0.326	0.28	2 %	0.235	0.235	0 %	0.184	0.179	0 %
A43	0.182	0.12	3 %	0.496	0.523	1 %	0.546	0.618	4 %
A44	0.128	0.128	0 %	0.135	0.134	0 %	0.159	0.137	1 %
A45	0.301	0.298	0 %	0.667	0.625	2 %	0.788	0.77	1 %
A46	0.27	0.294	1 %	0.313	0.304	0 %	0.323	0.345	1 %
A47	0.301	0.278	1 %	0.224	0.23	0 %	0.237	0.227	0 %
A48	0.166	0.159	0 %	0.139	0.166	1 %	0.231	0.232	0 %
A49	0.374	0.31	3 %	0.261	0.269	0 %	0.275	0.274	0 %

B: IL-6 concentration for all individuals

IL-6 concentration for all individuals at baseline, AHC and BMC shown on a log scale. Each coloured line represent one indivudual.



C: BMI

Table 3: BMI for all subjects at baseline, after AHC and after BMC. Missing data is marked with -.

	BMI		
Subject	Baseline	AHC	BMC
A	50,9	51,1	50,2
A3	43	42,4	42,3
A4	-	-	-
A8	44,6	44	43,5
A22	33,3	33	32,3
A23	30,2	29,8	0
A24	36,2	35,8	35,6
A26	37,2	36,9	36,7
A27	37,5	36,9	36,8
A28	31,7	31,5	31
A29	38,4	38,4	38,1
A30	31,1	30,7	30,1
A31	30,4	29,7	29,8
A32	32,1	32	31,5
A33	31,6	31,1	30,4
A35	35,1	35,2	34,9
A38	30,7	30,2	29,6
A39	32,3	32,1	31,5
A40	31,7	31,5	31,1
A41	30,5	29,9	29,4
A42	32,9	32,2	31,8
A43	33,3	32,5	31,9
A44	35,3	34,6	33,6
A45	35,5	35,1	34,5
A46	34,1	33,4	33,2
A47	32,5	32,2	31,8
A48	31,1	30,9	30,7
A49	33,1	32,7	31,9

D: CRP concentration

Table 4: CRP for all subjects at baseline, after AHC and after BMC. Missing data is marked with -.

	hs-CRP		
Subject	**Baseline**	**AHC**	**BMC**
A1	5.4	2.87	5.31
A3	5.83	7.86	7.04
A4	6.04	10.78	6.88
A8	22.77	21.92	18.52
A22	2.07	2.44	1.92
A23	11.53	18.55	6.93
A24	15.35	2.84	1.1
A26	4.51	9.29	10.14
A27	4.19	-	5.58
A28	3.39	0.88	0.49
A29	1.02	0.85	0.79
A30	2.06	2.11	1.74
A31	2.2	1.09	0.72
A32	6.82	7.66	7.57
A33	13.95	8.27	9.74
A35	3.99	4.64	6.33
A38	1.86	8.17	1.98
A39	3.62	2.43	6.47
A40	1.49	2.16	-
A41	0.92	0.49	1.14
A42	1.93	1.28	1.4
A43	2.24	2.98	3.72
A44	10.23	8.08	10.47
A45	1.77	1.73	2.09
A46	8.91	1.34	1.3
A47	10.05	11.21	6.31
A48	0.51	0.78	4.23
A49	7.72	6.66	5.67

E: Macronutrient composition of diets

Table 5: Diet macronutrient composition for diet AHC and diet BMC in E% [72].

	E%	
	AHC diet	**AHC diet**
Carbohydrate	65	27
Fibre	1.3	1.3
Protein	15	30
Total fat	20	43
SFA	3.75	9.50
MUFA	11.25	28.50
PUFA	5.0	5.0
n-6 PUFA	4.0	4.0
n-3 PUFA	1.0	1.0
n-3 PUFA (plant)	0.67	0.67
n-3 PUFA (fish)	0.33	0.33