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# Short-term high-fat overfeeding does not induce NF-κB inflammatory signaling in subcutaneous white adipose tissue

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**Abstract**

*Context:* It is unclear how white adipose tissue (WAT) inflammatory signaling proteins respond during the early stages of overnutrition.

*Objective:* To investigate the effect of short-term, high-fat overfeeding on fasting abdominal subcutaneous WAT total content and phosphorylation of proteins involved in nuclear factor-κB (NF-κB) inflammatory signaling, systemic metabolic measures and inflammatory biomarkers.

*Design:* Individuals consumed a high-fat (65% total energy total fat), high-energy (50% above estimated energy requirements) diet for 7 days.

*Results:* Fifteen participants (age 27 ± 1 y; BMI 24.4 ± 0.6 kg/m$^2$) completed the study. Body mass increased following high-fat overfeeding (+1.2 ± 0.2 kg; $P < 0.0001$). However, total content and phosphorylation of proteins involved in NF-κB inflammatory signaling were unchanged following the intervention. Fasting serum glucose (+0.2 ± 0.0 mmol/L), total cholesterol (+0.4 ± 0.1 mmol/L), low-density lipoprotein cholesterol (+0.3 ± 0.1 mmol/L), high-density lipoprotein cholesterol (+0.2 ± 0.0 mmol/L), and lipopolysaccharide-binding protein (LBP; +4.7 ± 2.1 µg/mL) increased, whereas triacylglycerol concentrations (-0.2 ± 0.1 mmol/L) decreased following overfeeding (all $P < 0.05$). Systemic biomarkers (insulin, soluble cluster of differentiation 14 (CD14), C-reactive protein, IL-6, TNF-α and monocyte chemoattractant protein-1) and the proportion and concentration of circulating CD14+ monocytes were unaffected by overfeeding.

*Conclusion:* Acute lipid oversupply did not impact on total content or phosphorylation of proteins involved in WAT NF-κB inflammatory signaling, despite modest weight gain and metabolic alterations. Systemic LBP, which is implicated in the progression of low-grade inflammation during the development of obesity, increased in response to a 7-day high-fat overfeeding period.

**Key words:** high-fat diet, white adipose tissue, inflammation, lipid profile, metabolic endotoxemia, monocytes

**Précis**

Healthy adults consumed a high-fat, high-energy diet for 7 days. The diet did not affect adipose tissue NF-κB inflammatory signaling, despite modest weight gain and some metabolic alterations.

## Introduction

An important factor underlying obesity and associated cardiometabolic diseases is chronic low-grade inflammation within metabolically active organs such as white adipose tissue (WAT) (1,2). Long-term positive energy balance can lead to WAT dysfunction through enhanced monocyte migration from the bloodstream into the tissue as a result of increased production of WAT-derived chemoattractant cytokines such as monocyte chemoattractant protein-1 (MCP-1) (1,3). Activation of the WAT toll like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) inflammatory signaling pathway can also lead to WAT dysfunction through increased inflammatory cytokine production, which act in a local and systemic manner to impair insulin sensitivity in the obese phenotype (3).

Examining the early inflammatory and metabolic responses to overnutrition in humans using short-term, high-fat overfeeding with SFA-rich foods may provide insight into cardiometabolic disease progression. Short-term (3- to 7-days) high-fat overfeeding has been found to impair insulin sensitivity and glycemic control in healthy individuals before substantial gains in body weight and fat mass are observed (4–7). We also recently reported that a 7-day period of lipid oversupply plays a role in the development of vascular inflammation and atherosclerosis (8). However, there is very little information on the initiation of inflammatory signaling pathways within WAT in response to acute overnutrition (9).

Previous high-fat overfeeding studies (3- to 56-days) have examined gene expression of targets involved in WAT inflammatory pathways, such as pro-inflammatory cytokines (IL-6, TNF-α and MCP-1), macrophage markers (cluster of differentiation (CD)68, CD11c and CD40), and components of the NF-κB pathway (10–14). However, measurements of gene

expression do not provide information on protein abundance, as post-transcriptional events such as translation and protein degradation are also involved in regulating protein levels (15). Thus, the amount and/or activity of proteins regulating inflammatory pathways may more accurately represent changes in the physiological function of WAT.

Overnutrition or the consumption of a high-fat diet (HFD) can also lead to a state of systemic metabolic endotoxemia as a result of gut-derived LPS translocating into the circulation (16,17). This too can initiate low-grade inflammation (18). The increase in LPS may be driven by dietary-induced impairments in gut barrier function (18) or its absorption may be facilitated alongside digested fats (19). The inflammatory effects of LPS are then mediated through its interactions with lipopolysaccharide-binding protein (LBP) and CD14 (20,21).

Further research is required to elucidate the complexities of WAT inflammatory signaling during the early stages of overnutrition. We investigated the effects of short-term, high-fat overfeeding on WAT total protein and phosphorylation of proteins involved in NF-κB inflammatory signaling (primary outcome), systemic metabolic and inflammatory biomarkers and circulating concentration of CD14+ monocytes (secondary outcomes). It was hypothesized that short-term high-fat overfeeding would initiate WAT NF-κB inflammatory signaling and alter systemic cardiometabolic risk markers. We performed biopsies of the abdominal subcutaneous WAT before and after 7 days of high-fat overfeeding in our healthy, non-obese adult cohort. This experimental model has been shown to induce a modest level of metabolic dysfunction in numerous previous studies (4–6). The abdominal subcutaneous WAT depot was chosen for the site of sampling as it has been reported to act as a proxy for visceral WAT inflammatory status in human obesity (22–25).

## Methods

### Study design and participants

Fifteen apparently healthy adults were recruited to participate in this open-label single-arm study that was conducted at the School of Sport, Exercise and Health Sciences, Loughborough University (United Kingdom) between July and November 2018. The nature of the study required an open investigation, with no blinding of the study to the participants or researchers. Participants were recruited to the study from Loughborough University and the surrounding area through posters, social media and noticeboard advertisements.

A favorable ethical opinion for conduct was given by Loughborough University Ethics Committee for Human Participants (Ethical Approval: R18-P040). The study was registered as a clinical trial (Clinicaltrials.gov ID: NCT03569189) and was conducted in accordance with the Declaration of Helsinki of 1975, as revised in 1983. Participants gave their written informed consent to participate prior to commencement of the study and after the experimental procedures, associated risks and potential benefits of participation were explained in full. All participants were free from cardiometabolic (including type 2 diabetes and cardiovascular diseases (CVDs)) or inflammatory disease, physically active (≥ 3 x 30 min moderate intensity exercise/wk), weight stable (< 3 kg gain or loss in last 3 months) and non-smokers [aged 18 - 40 y; BMI 21.0 - 29.9 kg/m$^2$]. Participants were also required to meet the following inclusion criteria: not taking medication/supplements known to interfere with the study outcomes; not prescribed antibiotics within the last 3 months; no food allergies/intolerances or eating habits that would impede compliance to the dietary intervention; not consuming excessive amounts of alcohol (men: < 21 units/wk; women: < 14 units/wk) and unrestrained eaters, as indicated by a dietary restraint score of < 11 on the Three-Factor Eating Questionnaire (Factor I) (26,27).

**Estimation of energy requirements**

Participants were required to report to the laboratory following a 12 h overnight fast on 3 occasions in total. In the first laboratory visit participants were screened. During the initial screening visit, after gaining informed consent, baseline anthropometric measurements were collected. Height and body mass were measured to the nearest 0.1 cm and 0.1 kg, respectively. Body mass (kg) and body fat (%) were assessed by bioelectrical impedance (Tanita BC-418MA) with the use of standard settings (normal body type and +0.5 kg for clothing). Waist circumference was measured from the midpoint between the lowest rib and the iliac crest and hip circumference was measured from the widest part of the hips. Waist and hip circumference were measured in triplicate and to the nearest 0.5 cm by the same researcher for all participants, an average value was then calculated. Information including participants' age, height, body mass and sex was then used to estimate individual resting energy expenditure (REE) according to calculations described by Mifflin *et al.* (28). A factor of 1.1 was applied to REE to account for the thermic effect of food and a standard correction for physical activity (PA) was employed (1.6 and 1.7 times REE for females and males, respectively) to estimate individual total daily energy requirements (5). This information was used to estimate each participant's energy intake (EI) requirements.

**Dietary protocol and adherence**

Diet was standardized for a 3-day period prior to the first experimental visit (baseline). This diet provided participants with 100% of their estimated required EI and was representative of the typical macronutrient intake of UK adults aged 19-64 y (29). Daily macronutrient intakes were targeted at 17.6% of total energy (%TE) protein, 34.7%TE total fat, 12.5%TE SFA, 47.6%TE carbohydrate and 19.0 g/d dietary fiber (% food energy values were chosen as

representative macronutrient intake targets since alcohol was not permitted during our dietary protocol) (29,30). Following a baseline (pre-intervention) experimental visit, participants consumed an experimental 7-day HFD, which provided an energy excess of 50% above estimated required EI, with daily macronutrient intakes targeted at 14.0%TE protein, 65.0%TE total fat, 32.5%TE SFA and 21.0%TE carbohydrate. Mean energy and macronutrient composition of the standardized diet provided at baseline and the HFD are outlined in **Table 1**. A post-intervention experimental visit was conducted the morning after completing the HFD. The experimental visit protocol is described in detail below. Commercially available foods were purchased and prepared by the research team at the metabolic kitchen in the School of Sport, Exercise and Health Sciences. The importance of consuming all of the food provided was emphasized to participants and they were instructed not to consume any other foods or nutritive beverages (including alcohol) during this time. The provided diets followed a normal daily feeding pattern (i.e. breakfast, lunch, dinner and snacks) and participants received daily food menus, which functioned as a reminder to consume all study meals and snacks. To encourage dietary adherence, a food preferences checklist was provided to participants prior to the intervention allowing a personalized diet to be formulated and ensuring individuals received only foods they were willing to consume. A wide variety of foods were included to minimise the likelihood of product boredom. When instructed to fry foods during the HFD, participants were asked to avoid wasting any left-over fat from the cooking process. Adherence to the diet was assessed by verbal communications at food collection visits. In the event of any uneaten foods, participants were instructed to notify the research team immediately and return these foods, so that alterative arrangements could be considered.

**Experimental visit protocol**

Experimental visits were conducted following a 12 h overnight fast before and after the 7-day period of high-fat overfeeding. The experimental protocol comprised of anthropometric measurements, a venous blood sample and a single, abdominal subcutaneous WAT biopsy. All participants were instructed to avoid strenuous PA in the 24 h preceding each experimental visit, compliance was assessed at experimental visits and participants were required to confirm they adhered to this protocol. All experimental visits were performed between 0700 and 1000 and were conducted at the same time of day for each participant.

**Adipose tissue biopsy collection**

WAT biopsies were obtained under sterile conditions by a trained researcher who remained consistent within trials for each participant. The tissue was collected from the lateral periumbilical region of the abdomen with a 5 mm Bergström type needle modified for use with suction (31). Biopsy samples taken before and after the intervention were taken from opposite sides of the umbilicus. To begin the procedure, the local area was thoroughly disinfected with Videne then anesthetized (1% Lidocaine Hydrochloride) before a horizonal ~1 cm incision was made 8-10 cm lateral to the umbilicus. The tissue sample was subsequently collected with the needle directed 4-5 cm in the medial direction. Immediately following collection, the sample was washed with saline over Nitex Nylon Fiber 250/50 (Plastok Associates Limited, UK) gauze and freed from any clots and connective tissue before being snap frozen in liquid nitrogen and stored at -80°C until analysis.

**Physical activity assessment**

All participants were required to maintain usual PA levels throughout the intervention. They were informed that a small amount of weight gain would be normal following the HFD intervention and were instructed not to attempt to offset this with additional PA. PA was

objectively measured for three consecutive days prior to each experimental visit i.e. during the 3-day dietary standardization period and for the final 3-day period of high-fat overfeeding. Participants wore an Actigraph accelerometer (GT3X + activity monitor; Actigraph, LLC) directly above the right hip during waking and sleeping hours, except during water-based activities. Valid monitor wear time was defined as achieving ≥ 600 min wear time/d, and non-wear time was defined as ≥ 60 min of consecutive zeros, allowing for 2 min of interruptions (32). PA data were analyzed in 60 s epochs (ActiLife Software, version 6.0). The Freedson MV3 combination algorithm was used to estimate energy expenditure (33). Average time spent in sedentary, light and moderate to vigorous physical activity (MVPA) were determined in accordance with standard cut points (34) and percentage of time/d spent in each activity intensity was calculated.

**Habitual dietary intake assessment**

Habitual dietary intake was assessed using a 3-day weighed food diary. Verbal and written instructions and a set of digital scales were provided to participants. The importance of accurate recording and not altering habitual dietary intake was emphasized. Participants completed the food diary over three consecutive days, including two weekdays and one weekend day. Upon completion, food diaries were assessed, and further detail was collected where necessary.

Dietplan software (version 7, Forestfield Software Ltd.) was used to calculate specific energy and macronutrient intake. The nutrient composition of foods was based on NDS Nutrient Database or McCance and Widdowson's (version 7) food tables. Dietary fiber intake was defined using the Association of Official Analytic Chemists (AOAC) method (30).

Determination of under-estimation of dietary EI was calculated for each participant. Basal metabolic rate (BMR), based on age, gender and body mass, was estimated using the Henry equation (35). An age specific mean physical activity level of 1.6 was assigned to represent the moderately physically active lifestyle of our population (36). The Goldberg lower 95% confidence limit was calculated to be 1.43 using the CV recommended by Black *et al.* (37). Participants with a ratio of reported EI:estimated BMR of < 1.43 were therefore identified as under reporters of EI (36).

**Biochemical analysis**

Venous blood samples were drawn into vacutainers containing either $K_3$-EDTA or a clotting catalyst for serum separation. (Greiner bio-one GmbH, Kremsmünster, Austria). Samples for serum collection were left at room temperature and samples for plasma collection were left on ice for 30 min before centrifugation at 2387 x *g* for 15 minutes at 4°C. Serum and plasma was subsequently extracted and immediately frozen at -80°C.

Serum samples were analyzed using commercially available spectrophotometric assays for glucose (#A11A01668), total cholesterol (TC; #A11A01634), HDL cholesterol (HDL-C; #A11A01636), triacylglycerol (TAG; #A11A01640) and C-reactive protein (CRP; #A11A01611), using a semi-automatic analyser (Pentra 400, Horiba Medical, Northampton, UK). The CV for serum glucose, TC, HDL-C, TAG and CRP was 0.5, 0.8, 0.8, 3.9 and 2.6%, respectively. The Friedewald equation was used to estimate LDL cholesterol (LDL-C) concentration (38). Serum insulin (EIA-2935, DRG Instruments GmBH, Marburg, Germany), LBP (Biometec, Greifswald, Germany), soluble CD14 (sCD14; R&D Systems, Minneapolis, MN, USA) and plasma IL-6, IL-1β, TNF-α and MCP-1 (R&D Systems, Minneapolis, MN, USA) concentrations were determined using ELISA, following manufacturer's instructions.

The minimal detectable concentrations were 0.04, 0.03, 0.02 and 1.7 pg/mL for IL-6, IL-1β, TNF-α and MCP-1, respectively. Plasma IL-6, TNF-α and MCP-1 concentrations were detectable in 100% of samples ($n$ = 15). At baseline and following overfeeding, IL-1β concentrations were only detectable in 3 and 4 participants, respectively. Therefore, we have not conducted statistical analysis on this outcome measure. The intra-assay CV for insulin, LBP, sCD14, IL-6, TNF-α and MCP-1 was 2.9, 6.9, 1.5, 6.9, 4.9 and 6.2%, respectively.

The HOMA-IR was calculated from fasting serum glucose and serum insulin concentrations (39), where glucose and insulin concentration units are mmol/L and µIU/mL, respectively:

$$HOMA - IR = \frac{glucose\ x\ insulin}{22.5}$$

**Peripheral blood mononuclear cell isolation and flow cytometry analysis**

Differential white blood cell count, including monocytes was obtained using a Yumizen H500 cell counter (Horiba Medical, Montpellier, France) from $K_3$-EDTA-treated whole blood. Peripheral blood mononuclear cells (PMBC) were then isolated from $K_3$-EDTA treated whole blood by density gradient centrifugation and cryopreserved until future immunophenotyping. To isolate PMBCs, whole blood was diluted 1:1 with 1 x phosphate buffered saline (PBS) and then layered on Histopaque-1077 (Sigma, Dorset) before centrifugation at 400 x $g$ for 40 min at 19°C. The PBMC layer was aspirated and washed twice with PBS by centrifugation steps at 300 x $g$ for 10 min at 19°C. PBMC pellets were then stored in 2 x 1 mL aliquots in 61.6% RPMI medium, 27% fetal bovine serum (FBS), 10% DMSO, 0.7% penicillin-streptomycin 10000 U/mL, 0.7% L-Glutamine 200 mM and frozen at a rate of -1°C/min to -80°C. Then, samples were transferred to liquid nitrogen and stored until subsequent analysis by flow cytometry.

Cryopreserved PBMCs were rapidly thawed in 1 mL of 89.5% FBS, 10% reaction buffer (100mM Tris-HCl (pH 7.5), 25mM $MgCl_2$, 1mM $CaCl_2$), 0.5% DNase 1 U/mL at 37°C for 3 min. Once thawed, cells were washed twice in complete media (76.95% RPMI, 20% FBS, 2% penicillin streptomycin 5000 U/mL, 1% L-glutamine 200 mM, 0.05% DNase 1 U/mL). PBMCs were counted using CountBright absolute counting beads (ThermoFisher Scientific, #C36950) on a BD C6 Accuri Flow Cytometer (Becton Dickinson, Oxford, UK) and 175,000 cells were used for subsequent antibody staining. Prior to antibody staining, cells were washed in 1 mL FACS buffer (Dulbecco's PBS supplemented with 1% bovine serum albumin (BSA), 2mM EDTA and 0.1% sodium azide) and centrifuged at 300 x *g* for 5 min. Cells were then incubated in the dark for 30 min at room temperature in FACS buffer with 10 µL fluorescein isothiocyanate-conjugated CD14, clone M5E2 (BD Biosciences, #555397) antibody and 5 µL 7-AAD (BD Biosciences, #559925). Cells were then washed twice with FACS buffer for 5 minutes at 300 x *g* and cells were resuspended in 200 µL FACS buffer prior to acquisition.

Flow cytometry data were analyzed using BD Accuri software (Becton Dickinson, Oxford, UK). Briefly, forward versus side scatter was used to gate on the monocyte population by morphology. Doublets were discriminated using FSC-A versus FSC-H plots and non-viable monocytes (7-AAD+) were excluded. The proportion (%) of CD14+ monocytes within the total monocyte population was then established. An example of the gating strategy used can be seen in **Figure 1**. The circulating concentration of CD14+ monocytes was calculated by applying this proportion to the total monocyte count derived from the hematology analyzer. Compensation was adjusted monthly by using single stained controls and gates established using fluorescence minus one controls.

**Adipose tissue protein content determination**

Adipose tissue samples (~75 mg) were homogenised in 2.5 µL/mg buffer (1 x PBS containing 1% Triton X-100, 1% protease and phosphatase inhibitor cocktail (Halt protease and phosphatase inhibitor cocktail 100X; Thermo Fisher Scientific, Rockford, IL, USA), and 1% EDTA) using a tissue lyser (TissueLyser II) and 5 mm stainless steel beads. Samples were then placed on a tube rotator for 30 min at 4°C. Then, homogenates were centrifuged, and the protein content of the supernatant was determined using the Pierce 660 nm protein assay (Thermo Scientific, Rockford, IL, USA). Following protein determination, adjusted volumes of each homogenate (to provide 60 µg of total protein) was prepared using a commercially available clean-up kit (ReadyPrep 2-D Clean-up Kit, Bio-Rad), used in accordance with the manufactures' instructions. The protein pellet resulting from the clean-up kit was resuspended in 60 µL of 1 x LDS (NuPAGE 4x LDS diluted to 1x in distilled water; Invitrogen, Carlsbad, CA, USA) containing 5% β-mercaptoethanol and incubated at room temperature for 5 min before boiling at 95°C for 10 min. The samples were subsequently stored at -20°C until analysis.

Protein extracts (5 µg) were loaded onto NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and separated before transfer to polyvinylidene (PVDF) membranes (Invitrogen, Carlsbad, CA, USA). Membranes were then blocked for non-specific binding (Tris-buffered saline with 0.1% Tween 20 (TBST) and 5% non-fat dry milk (NFDM) or 5% BSA) for 1 h at 4°C. Following this, membranes were washed in TBST for 3 x 5 min with gentle agitation before overnight incubation at 4°C in TBST and 2% NFDM with primary antibodies against TLR4 (1:2500; Santa Cruz Biotechnology; SC 293072), NF-κB p65 (1:2500; Cell Signaling Technology; #4764), phosphorylated (p)-NF-κB p65$^{ser536}$ (1:500; Cell Signaling Technology;

#3033), Inhibitory factor κBα (IκBα) (1:2500; Cell Signaling Technology; #4812), p-IκBα$^{ser32}$ (1:1000; Abcam; Ab92700), P38 (1:1000; Cell Signaling Technology; #9212), p-P38$^{Thr180/Tyr182}$ (1:1000; Cell Signaling Technology; #4511) and β-Actin (1:2500; Cell Signaling Technology; #4967) or TBST and 2% BSA with primary antibody against MCP-1 (1:1000; Cell Signaling Technology; #2027). Thereafter, membranes were washed in TBST for 3 x 5 min with gentle agitation before incubation for 1 h at room temperature with an appropriate horseradish peroxidase-conjugated secondary antibody (1:2000; Cell Signaling Technology; #7074 or #7076) in TBST and 2% NFDM or 2% BSA. Following this, membranes were washed in TBST for 3 x 5 min. The bands were then visualized by means of an enhanced chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL, USA). Images were taken using a molecular imager ChemiDoc XRS+ (Bio-Rad Laboratories, Richmond, CA, USA) and quantified using Quantity One image-analysis software (version 4.6.8; Bio Rad Laboratories, Richmond, CA, USA).

**Statistical analyses**

The primary outcome measure of this study was the total content and phosphorylation of proteins involved in WAT NF-κB inflammatory signaling. Secondary outcome measures were: fasting indexes of insulin resistance (glucose, insulin, HOMA-IR); lipid profile (TC, LDL-C, HDL-C, TAG); metabolic endotoxemia (LBP, sCD14); systemic inflammation (CRP, IL-6, TNF-α, MCP-1); and the concentration and proportion of circulating CD14+ monocytes.

A statistical power calculation of pilot data from our laboratory (data not presented) was conducted using G*Power (version 3.1, Germany). This study required 11 participants to detect a significant change in the activation of NF-κB in WAT, with a power of 80% and an α

error probability of 0.05. To allow for a 25% drop-out rate, 15 participants were enrolled onto the study.

The difference in outcome measures before and after 7-day of high-fat overfeeding were assessed with Student's paired samples *t*-tests when normal distribution was achieved, as defined by the Shapiro-Wilk test. When normality was not achieved, significance was assessed using the Wilcoxon signed-rank test. All statistical analyses were performed using SPSS (Version 23), using a per-protocol analysis approach. All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. Statistical significance was accepted when $P < 0.05$.

## Results

### Participants

Participant flow through the study is illustrated in **Figure 2**. Of the 66 individuals assessed for eligibility, 15 participants were allocated to intervention and completed the study. One participant was removed from the analyses of the primary outcome (i.e. WAT NF-κB inflammatory signaling) as no post-intervention visit biopsy was collected; this was due to prolonged bleeding from the biopsy site following sample collection during the pre-intervention experimental visit. The baseline characteristics of the participants are reported in **Table 2**.

### Dietary compliance, body mass and physical activity

The HFD was well tolerated by all participants and no adverse events were reported. Participants reported achieving 100% and 99.97% compliance for consuming all study meals and snacks provided during the dietary standardization and the HFD period, respectively. All

participants gained body mass following high-fat overfeeding (Pre: 75.2 ± 2.9 kg; Post: 76.4 ± 2.9 kg; $P < 0.0001$). Activity energy expenditure and percentage of time spent sedentary and in light or MVPA were not statistically different during the 3-day period prior to each experimental visit (**Table 3**). Participant compliance was 100% for meeting valid wear time criteria of ≥ 600 min. Due to a technical error with a device on one monitoring occasion, data were analyzed for $n = 14$.

**Habitual dietary intake and energy requirements**

The energy and macronutrient composition of reported habitual dietary intake are presented in **Table 1**. Based on the assumption that participants were in energy balance for the habitual dietary monitoring period, recorded habitual EI was underestimated by 33% of participants ($n = 5$) (36,37). There was a significant difference between EI recorded during habitual monitoring and planned EI for the standardized diet when all participants data ($n = 15$) were analyzed (9996 ± 1006 kJ vs. 12648 ± 566 kJ, respectively; $P = 0.023$). However, when the under-reporters ($n = 5$) were excluded from analysis, there was no significant difference between EI recorded during habitual monitoring and the period of dietary standardization for 10 participants (11523 ± 1188 kJ vs. 11981 ± 718 kJ, respectively; $P = 0.193$). This supported the use of employing an estimated energy requirement (28) for the dietary standardization and HFD period within this study, rather than relying on reported habitual EI to inform such calculations.

**Fasting systemic metabolic and inflammatory biomarkers**

The effect of high-fat overfeeding on fasting systemic biomarkers are presented in **Table 4**. Fasting serum glucose, TC, LDL-C, HDL-C, LBP and LBP:sCD14 ratio significantly increased, whereas TAG decreased following high-fat overfeeding. Fasting insulin, HOMA-

IR, TC:HDL ratio, LDL-C:HDL-C ratio, CRP, sCD14, IL-6, TNF-α and MCP-1 were unaffected by high-fat overfeeding.

**Adipose tissue intracellular signaling**

Due to the lack of a post intervention WAT biopsy for one participant, pre- and post-HFD samples were analyzed for 14 participants. The total protein content, phosphorylation and ratio of phosphorylation to total protein content of P38, NF-κB and IκBα are presented in **Figure 3** (A-I) and total protein content of TLR4 and MCP-1 are presented in **Figure 4** (A and B). Representative blots for each protein analyzed are presented in **Figure 5**.

There was no change in response to high-fat overfeeding for the total protein content, phosphorylation or ratio of phosphorylation to total protein content of P38, NF-κB or IκBα. Total protein content of TLR4 and MCP-1 was also unchanged following high-fat overfeeding.

**Proportion and concentration of CD14+ monocytes**

Due to sample collection and storage issues beyond our control, pre- and post-HFD samples were analyzed for 9 participants. Monocyte cell viability determined by 7-AAD staining was 98.4 ± 0.2%. There was no change in the whole blood monocyte count (Pre: 0.46 ± 0.04 $10^9$/L; Post: 0.52 ± 0.05 $10^9$/L, $P$ = 0.274), the percentage of CD14+ monocytes within the total monocyte population (Pre: 84.4 ± 1.8%; Post: 84.0 ± 2.4%, $P$ = 0.794) or in the concentration of CD14+ monocytes (Pre: 391 ± 41 cells/μL; Post: 438 ± 49 cells/μL, $P$ = 0.318) following high-fat overfeeding.

**Discussion**

Interpreting early WAT inflammatory responses to overnutrition is important as it may help to increase understanding of the development of obesity and cardiometabolic disease progression. To our knowledge, this is the first study to illustrate that feeding a HFD to healthy, non-obese adults at 1.5 times their estimated energy intake for a 7-day period had no impact on the content or phosphorylation of selected proteins involved in WAT NF-κB inflammatory signaling, despite a modest increase in body mass (+1.2 kg) and alterations in several systemic biomarkers of cardiometabolic health.

Previous dietary interventions conducted in overweight cohorts found an increased expression of WAT inflammatory genes in response to a SFA-rich 56-day isocaloric diet (40) (19%TE SFA, 11%TE MUFA) and 21-day SFA-rich overfeeding diet (13) (where habitual diet was supplemented with an additional 4184 kJ/day from coconut oil, butter and blue cheese). This led to our hypothesis that short-term high-fat overfeeding (with foods typically high in SFAs, including processed meats, butter, cheese, savory snacks and pastries) would initiate WAT NF-κB inflammatory signaling. Contrary to our expectations, we demonstrated that modest weight gain induced by a 7-day period of high-fat overfeeding occurred without modification of total protein and phosphorylation of proteins involved in the WAT NF-κB inflammatory signaling pathway in healthy, lean adults.

In previous high-fat overfeeding studies (3- to 56-days), healthy adults were provided with an overfeeding diet or supplemented their habitual diet with high-fat snacks, desserts and oil-based supplements or foods (butter, almonds, cheese) to increase EI by 3180 to 5230 kJ/day above requirements (total dietary fat intake: 45-48%TE) (10–12,14). Despite average weight gain in these cohorts ranging from 0.7 - 7.6 kg, high-fat overfeeding led to no change in genes

related to WAT inflammation, including IL-10 (11), MCP-1 (10–12), NF-κB, IL-6, IL-10 or adiponectin (12). Some support for a possible WAT inflammatory response to 14- and 56-day high-fat overfeeding was reported by Alligier *et al.* (14), who observed increased WAT gene expression of LBP, which is implicated in the activation of the TLR4 inflammatory signaling pathway (20) and may act as an early indicator of inflammation and obesity-related WAT dysfunction (41). The novelty of our work is that rather than measuring changes in gene expression, that do not necessarily translate into meaningful information regarding protein abundance (15), we have measured the protein content and phosphorylation (an activity readout) of proteins involved in the NF-κB signaling pathway. Due to tissue quantity limitations, it was not possible for us to assess the impact of our intervention on additional inflammatory outcomes, such as the gene expression of pro-inflammatory cytokines. Nonetheless, our observation that a 7-day high-fat overfeeding period did not influence the content or phosphorylation of selected inflammatory signaling proteins, along with the aforementioned studies that typically did not observe changes in gene expression of inflammatory markers over a timeframe of 3- to 56-d (10–12,14), suggest that WAT inflammation may be secondary to the development of obesity. These observations are consistent despite the use of feeding interventions that varied in duration, severity (i.e. energy, total fat or SFA content) and food sources. Our findings, along with the other human intervention studies mentioned above, relate to subcutaneous WAT only so it is possible that other fat depots, such as visceral WAT, may have been affected by high-fat overfeeding. High-fat feeding in mice was found to induce macrophage infiltration and enhance inflammatory gene expression in epididymal (visceral) WAT, by as early as 3 days (42). In that study, systemic insulin resistance was found to worsen alongside increasing adipose tissue inflammation, as HFD exposure continued (42). Depleting macrophages using genetic and pharmacological methods did not attenuate the early stages of insulin resistance (42).

However, these methods were effective in ameliorating insulin resistance at 14 weeks of HFD exposure, at a time when severe obesity was established (42). In agreement with data from another rodent study (43), these findings indicate that visceral WAT inflammation contributes towards the maintenance of diet-induced insulin resistance during established obesity but that it is not an early driver of HFD-induced insulin resistance. However, caution is needed when extrapolating rodent data into humans as there are anatomical and functional differences between the species (44).

We observed no change in the proportion or concentration of CD14+ monocytes (primarily classical monocytes) in response to high-fat overfeeding. Classical monocytes (~80% of monocyte pool) have the highest propensity to migrate into WAT through their high C-C chemokine receptor type 2 (CCR2) receptor expression and interaction with complementary WAT-derived ligand MCP-1 (45,46). In the present study, no change in WAT MCP-1 total protein content was observed, along with no change in circulating MCP-1 concentration, which supports the unaltered proportion and concentration of CD14+ monocytes. In agreement with our data, no change in the number of circulating monocytes or WAT macrophage number was reported following 28-day overfeeding in normolipidemic, non-diabetic individuals (11), suggesting that monocyte migration into WAT does not significantly alter during overfeeding of this duration (< 28 day).

Metabolic endotoxemia has also been linked to the development of low-grade inflammation in the early stages of obesity (16,17). LBP and sCD14 are clinical measures of metabolic endotoxemia that reflect systemic exposure to LPS (47). More specifically, LBP is related to the pro-inflammatory actions of LPS (48), whereas sCD14 transfers LPS to plasma lipoproteins and promotes inhibition of cytokine production (49). In agreement with the

present investigation, LBP:sCD14 ratio was increased following 21-day SFA-rich (13) and 56-d high-fat (50) overfeeding interventions, although LBP and sCD14 were unaltered in the latter study (50). Findings from some studies suggest that the LBP:sCD14 ratio may be a better indicator of endotoxin exposure than either marker in isolation (50,51). The elevated LBP:sCD14 ratio that we observed following overfeeding was primarily driven by a significant increase in LBP concentration. Feeding mice high-fat diets varying in FA composition was shown to have differential effects on the circulating LBP:sCD14 ratio (51). A high-fat diet enriched in palm oil (which is most comparable to the westernized HFD employed in our study) increased LBP (and led to the highest LBP:sCD14 ratio), whereas the rapeseed oil diet was shown to dampen the pro-inflammatory outcomes of the HFD through an increase in the sCD14 concentration (51). Additionally, data from a human model of experimental overnutrition indicated that pro-inflammatory status (as assessed by plasma IL-6 concentrations) was linked to relative variations in LBP and sCD14 concentrations during the initial stages of weight gain (50). The absence of any change in CD14+ monocyte numbers along with no changes in WAT NF-κB signaling, despite an increase in LBP, could mean that a greater increase in LBP is necessary to initiate inflammatory pathways. It is also possible that the diet-induced increase in LBP may have activated inflammatory pathways in other WAT depots, such as visceral fat, but this is speculative and not that likely given that we observed no other changes in systemic markers of inflammation.

Consistent with our previous studies (4–6), we observed an increase in fasting glucose concentrations in response to high-fat overfeeding. The increase in glucose concentration after high-fat overfeeding has been attributed to reduced clearance by peripheral tissues (including skeletal muscle) (6,7). We did not observe a significant increase in insulin or HOMA-IR (a marker of insulin resistance) in response to overfeeding in our lean, metabolically healthy

cohort. Findings from a series of studies by Shimobayashi *et al.* indicated that visceral WAT inflammation is a secondary phenomenon, which occurs in response to insulin resistance (52). Although Luukkonen and colleagues found a 23% increase in HOMA-IR and increases in genes related to nucleotide-binding oligomerization domain-like receptor signaling in response to 21-day SFA-rich overfeeding in a cohort of overweight/obese individuals (13), our findings add weight to the suggestion that WAT inflammation may occur subsequent to weight gain and insulin resistance (11,12,14,53). The modifications in the lipid profile that we observed following our SFA-rich HFD (mainly derived from animal sources) agree with findings from a meta-analysis which indicate that an increased intake of SFAs (including myristic and palmitic acid) raise serum TC, LDL-C, HDL-C, and lower TAG concentrations, compared to carbohydrates (54). HDL-C has been suggested to be an acute-phase reactant, produced in response to local inflammation in the gut (55,56) and thus may be linked to the increase in LBP concentration observed in the present study. Elevated HDL-C has been associated with increased gut permeability and serum LPS in healthy males (55), which is interesting as low HDL-C levels have been traditionally linked to increased CVD risk (57). However, the FA composition of obesogenic HFDs may have a pivotal role to play in HDL functionality and may differentially affect CVD risk through alteration of liver-to-feces reverse cholesterol transport (58). Indeed, feeding mice a HFD rich in SFAs led to the enrichment of the proteome of HDL particles with pro-inflammatory hepatic-derived acute-phase proteins, compared to a MUFA-rich HFD (58). Our observation of a significant reduction in fasting TAG concentrations in response a high-fat overfeeding is consistent with previous work by our group (4–6) and that of others (59); this response could be partly explained by a reduction in endogenous TAG reduction in response to an acute increase in total fat intake (60).

A strength of the present study was the well-controlled nature of the dietary intervention. However, participants recruited were healthy, predominantly white, lean individuals. Therefore, our findings are not generalizable across different groups including obese populations, who may have differential WAT molecular responses to short-term overfeeding (61). Our study design did not include a control arm with habitual diet maintenance. This design choice was made for ethical reasons and allowed us to minimize the number of participants required to undergo invasive biopsy procedures. In line with previous hyperenergetic HFD human studies, subcutaneous WAT offered the possibility of sampling that is tolerable for a non-patient cohort undergoing an experimental study (10–14,40). It is therefore not possible for us to comment on the potential impact of our intervention on inflammation in the visceral WAT depot. The accumulation of visceral WAT, in addition to subcutaneous abdominal WAT that was sampled in the present study, leads to a higher risk of T2D and CVD development (62,63). However, visceral WAT exhibits the greatest link with inflammation and risk factors for the development of cardiometabolic disease (64–66). Human studies of obesity have indicated that there may be depot-specific differences in inflammatory activity, including elevated expression of inflammatory cytokines and/or a higher number of macrophages in visceral vs. subcutaneous WAT (67–69). We chose to sample abdominal subcutaneous WAT as it has been shown to act as a reasonable proxy for visceral WAT inflammation in human obesity (22–25). Clinical human data indicates that an increase in adiposity was correlated with changes in metabolic and immunoregulatory molecular adaptations in both visceral and abdominal subcutaneous WAT that are associated with fat mass and indicators of insulin sensitivity (70). It could be speculated that our biopsy procedure provoked an acute psychosocial stress response in our participants, but it is likely that this would have had minimal impact on our outcome measures. For example, Richlin *et al*. (2004) demonstrated that despite acute stress-induced enhancement of NF-κB DNA-

binding activity in the circulating PBMC pool (< 10 min following sympathetic nervous system activation), these effects were not mediated by the CD14+ monocyte fraction (71).

In conclusion, the total protein content or phosphorylation of proteins involved in WAT NF-κB inflammatory signaling was unaffected by a short-term, high-fat energy surplus in healthy, non-obese adults, despite a modest increase in body mass and alterations in systemic biomarkers of cardiometabolic health. We show that systemic LBP, a marker of metabolic endotoxemia, increased in response to a 7-day period of high-fat overfeeding. This finding supports data which suggests that LBP is implicated in the progression of low-grade inflammation during the initial stages of high-fat diet-induced obesity (16).

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**Author contributions**

OM and CJH conceived the research idea. AJW, LBS and NCB assisted with designing the study (development of overall research plan). RD-T, RMW, CJH and OM designed the experimental feeding model. RD-T, CJH and OM conducted the research (hands-on conduct of the experiments and data collection). RD-T, AJW, RMW, CJH and OM analyzed data or performed statistical analysis. RD-T, CJH and OM wrote the manuscript, with contributions from all co-authors. OM had primary responsibility for final content. All authors read and approved the final manuscript. The funding agencies played no role in the design, analysis, or data interpretation of the study.

**Data availability**

The datasets generated during the current study are not publicly available but are available from the corresponding author on reasonable request.

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## TABLES

**Table 1.** Energy and macronutrient composition of the 3-day habitual intake, 3-day dietary standardization and the 7-day high-fat, overfeeding period

| | Habitual diet | Dietary standardization | High-fat diet |
|---|---|---|---|
| Energy, kJ/d | 9996 ± 1006 | 12648 ± 566 | 18977 ± 854 |
| Protein | | | |
| g/d | 107.2 ± 8.5 | 133.4 ± 6.0 | 158.5 ± 7.1 |
| %TE | 18.6 ± 1.1 | 17.6 ± 0.0 | 14.0 ± 0.0 |
| Total fat | | | |
| g/d | 93.0 ± 11.9 | 116.7 ± 5.2 | 327.0 ± 14.8 |
| %TE | 34.3 ± 1.6 | 34.7 ± 0.0 | 64.9 ± 0.0 |
| Saturated fatty acids | | | |
| g/d | 33.5 ± 3.8 | 42.6 ± 2.1 | 164.8 ± 7.5 |
| %TE | 12.6 ± 0.8 | 12.6 ± 0.1 | 32.7 ± 0.1 |
| Monounsaturated fatty acids | | | |
| g/d | 34.5 ± 5.8 | 53.8 ± 2.2 | 116.7 ± 5.2 |
| %TE | 12.4 ± 0.8 | 16.1 ± 0.1 | 23.2 ± 0.1 |
| Polyunsaturated fatty acids | | | |
| g/d | 14.0 ± 2.1 | 14.9 ± 0.7 | 29.9 ± 1.4 |
| %TE | 5.2 ± 0.3 | 4.4 ± 0.1 | 5.9 ± 0.1 |
| *Trans* fatty acids | | | |
| g/d | 1.0 ± 0.2 | 1.5 ± 0.2 | 4.3 ± 0.2 |
| %TE | 0.4 ± 0.1 | 0.2 ± 0.0 | 0.4 ± 0.0 |
| Carbohydrate | | | |

| | | | |
|---|---|---|---|
| g/d | 275.2 ± 28.2 | 359.8 ± 16.1 | 239.6 ± 10.7 |
| %TE | 46.4 ± 1.7 | 47.6 ± 0.0 | 21.1 ± 0.0 |
| Total sugar | | | |
| g/d | 94.2 ± 10.7 | 91.2 ± 3.2 | 37.0 ± 2.3 |
| %TE | 16.2 ± 1.3 | 12.5 ± 0.7 | 3.2 ± 0.1 |
| Free sugars | | | |
| g/d | 40.2 ± 7.5 | 57.5 ± 2.1 | 35.3 ± 2.6 |
| %TE | 6.5 ± 1.1 | 7.8 ± 0.4 | 3.1 ± 0.2 |
| Dietary fiber (AOAC) | | | |
| g/d | 26.2 ± 3.4 | 19.6 ± 0.4 | 17.3 ± 0.7 |
| Alcohol[a] | | | |
| g/d | 11.8 ± 2.6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| %TE | 3.3 ± 0.7 | 0.0 ± 0.0 | 0.0 ± 0.0 |

Values are mean ± SEM, *n* = 15. Abbreviations: AOAC, Association of Official Analytical Chemists; %TE, percentage of total energy.

[a]Alcohol intake was not permitted during the dietary standardization or the high-fat diet period.

**Table 2.** Baseline characteristics of participants that completed the investigation

| Parameter | |
|---|---|
| Gender m(f) [a], *n* | 9(6) |
| Age[2], y | 27 ± 1 |
| Body mass[a], kg | 75.1 ± 2.9 |
| Ethnicity[a], *n* (%) | |
| White | 14 (93.3) |
| Mixed (White and Asian) | 1 (0. 7) |
| BMI[2], kg/m$^2$ | 24.4 ± 0.6 |
| Body fat, % | 21.4 ± 1.8 |
| Waist circumference[a], cm | 82.1 ± 1.7 |
| Hip circumference[a], cm | 103.3 ± 1.9 |
| Systolic blood pressure[a], mmHg | 119 ± 3 |
| Diastolic blood pressure[a], mmHg | 66 ± 2 |
| Fasting serum biomarkers | |
| Glucose, mmol/L | 4.4 ± 0.1 |
| Insulin, pmol/L | 75.3 ± 6.0 |
| TC, mmol/L | 4.5 ± 0.2 |
| LDL-C, mmol/L | 2.6 ± 0.2 |
| HDL-C, mmol/L | 1.5 ± 0.1 |

| | |
|---|---|
| TAG, mmol/L | 0.9 ± 0.1 |
| Dietary restraint score[a,b] | 7.9 ± 0.6 |

Values presented as mean ± SEM, *n* = 15. Abbreviations: BMI, Body mass index; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; TAG, triacylglycerol; TC, total cholesterol.

[a]Data collected at screening visit.

[b]Quantified by using the Three-Factor Eating Questionnaire (Factor I) (26). A score of < 11/21 indicated that an individual was classified as a non-restrained eater (27).

**Table 3.** Physical activity energy expenditure and percentage of time spent in physical activity intensities during the 3-day dietary standardization period (Pre) and for the final 3-day period (Post) of high-fat overfeeding

| | Pre | Post | *P* value |
|---|---|---|---|
| Accelerometer, counts/min | 357 ± 32 | 322 ± 27 | 0.280 |
| Activity energy expenditure, kJ/d | 2390 ± 191 | 2149 ± 203 | 0.291 |
| Monitor wear time, min/d | 1054 ± 42 | 1033 ± 38 | 0.515 |
| Activity intensity, | | | |
| Sedentary time, % activity wear time/d | 67.1 ± 2.3 | 68.9 ± 2.6 | 0.240 |
| Light PA, % activity wear time/d | 28.0 ± 2.2 | 26.7 ± 2.5 | 0.380 |
| Moderate to vigorous PA, % activity wear time/d | 4.9 ± 0.7 | 4.4 ± 0.7 | 0.295 |

Values are mean ± SEM, $n = 14$. All *P* values calculated using Student's paired samples *t*-tests. Abbreviation: PA, physical activity.

**Table 4.** Fasting systemic metabolic and inflammatory biomarkers before (Pre) and after

| | Pre | Post | *P* value |
|---|---|---|---|
| Glucose, mmol/L | 4.4 ± 0.1 | 4.6 ± 0.1 | 0.022[a] |
| Insulin, pmol/L | 75.3 ± 6.0 | 79.0 ± 8.7 | 0.804[a] |
| HOMA-IR | 2.5 ± 0.2 | 2.7 ± 0.3 | 0.382[a] |
| TC, mmol/L | 4.5 ± 0.2 | 5.0 ± 0.2 | <0.0001 |
| LDL-C, mmol/L | 2.6 ± 0.2 | 2.9 ± 0.2 | 0.001 |
| HDL-C, mmol/L | 1.5 ± 0.1 | 1.7 ± 0.1 | <0.0001 |
| TC:HDL-C ratio | 3.1 ± 0.2 | 2.9 ± 0.1 | 0.055[a] |
| LDL-C:HDL-C ratio | 1.8 ± 0.2 | 1.7 ± 0.1 | 0.202 |
| TAG, mmol/L | 0.9 ± 0.1 | 0.7 ± 0.0 | 0.007 |
| CRP, mg/L | 0.8 ± 0.3 | 5.8 ± 4.0 | 0.135[a] |
| LBP, µg/mL | 12.9 ± 1.3 | 17.7 ± 3.0 | 0.030[a] |
| sCD14, µg/mL | 1.47 ± 0.04 | 1.46 ± 0.06 | 0.776 |
| LBP:sCD14 ratio | 8.74 ± 0.83 | 11.70 ± 1.62 | 0.014 |
| IL-6, pg/mL | 1.37 ± 0.60 | 2.22 ± 0.67 | 0.211[a] |
| TNF-α, pg/mL | 0.93 ± 0.09 | 0.90 ± 0.06 | 0.516 |
| MCP-1, pg/mL | 176 ± 10 | 173 ± 9 | 0.637 |

(Post) a 7-day period of high-fat overfeeding

Values presented as mean ± SEM, *n* = 15 for all values. *P* values calculated using Student's paired samples *t*-tests, unless otherwise stated.

[a]*P* value was calculated with the Wilcoxon signed-rank test. Abbreviations: CRP, C-reactive protein; HOMA-IR, homeostatic model of insulin resistance; HDL-C, HDL cholesterol; LBP, lipopolysaccharide-binding protein; LDL-C, LDL cholesterol; MCP-1, monocyte chemoattractant protein-1; sCD14, soluble CD14; TAG, triacylglycerol; TC, total cholesterol.

**Figure Legends**

**Figure 1.** Gating strategy to identify CD14+ monocytes. From peripheral blood mononuclear cells, the monocyte population was identified by forward vs side scatter (A) and then doublets (B) and non-viable (7-AAD+) monocytes (C) excluded. The CD14+ gate was established for each participant using a fluorescence minus one control (D) and then the percentage of CD14+ monocytes determined (E).

**Figure 2.** Flow of participants through the different stages of the study. Abbreviations: HFD, high-fat diet; WAT, white adipose tissue.

**Figure 3.** Total protein, phosphorylation and the ratio of phosphorylated to total protein content of targets involved in the white adipose tissue nuclear factor–κB (NF- κB) inflammatory pathway before (Pre) and after (Post) a 7-day period of high-fat overfeeding (A-I). Each dot represents the individual response of a study participant. The bold line represents the mean response for $n$ = 14. Error bars represent SEM. Data were normalized to β Actin. $P$ > 0.05 for all Pre- vs. Post high-fat overfeeding comparisons. Abbreviations: AU, arbitrary units; IκBα, inhibitory factor κBα; NF-κB, nuclear factor-κB; p-, phosphorylated.

**Figure 4.** Total protein content of TLR4 (A) and MCP-1 (B) in white adipose tissue before (Pre) and after (Post) a 7-day period of high-fat overfeeding. Each dot represents the individual response of a study participant. The bold line represents the mean response for $n$ = 14. Error bars represent SEM. Data were normalized to β Actin. $P$ > 0.05 for all Pre- vs. Post

high-fat overfeeding comparisons. Abbreviations: AU, arbitrary units; MCP-1, monocyte chemoattractant protein 1; TLR4, toll like receptor 4.

**Figure 5.** Representative Western Blot images of the analyzed proteins and phosphorylation (p) sites of proteins involved in white adipose tissue inflammation pathway before (Pre) and after (Post) a 7-day period of high-fat overfeeding.

Figure 1

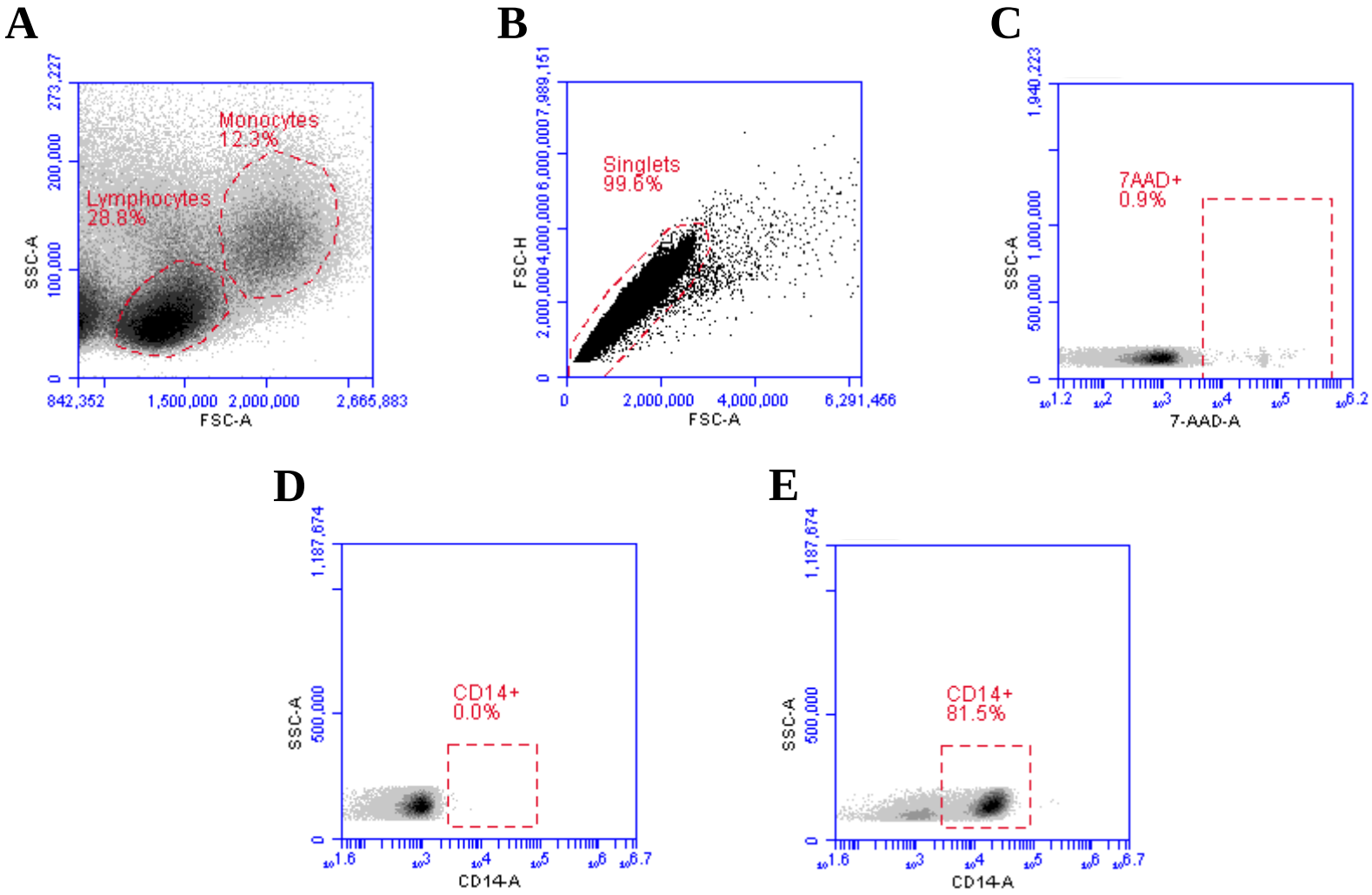

Figure 2

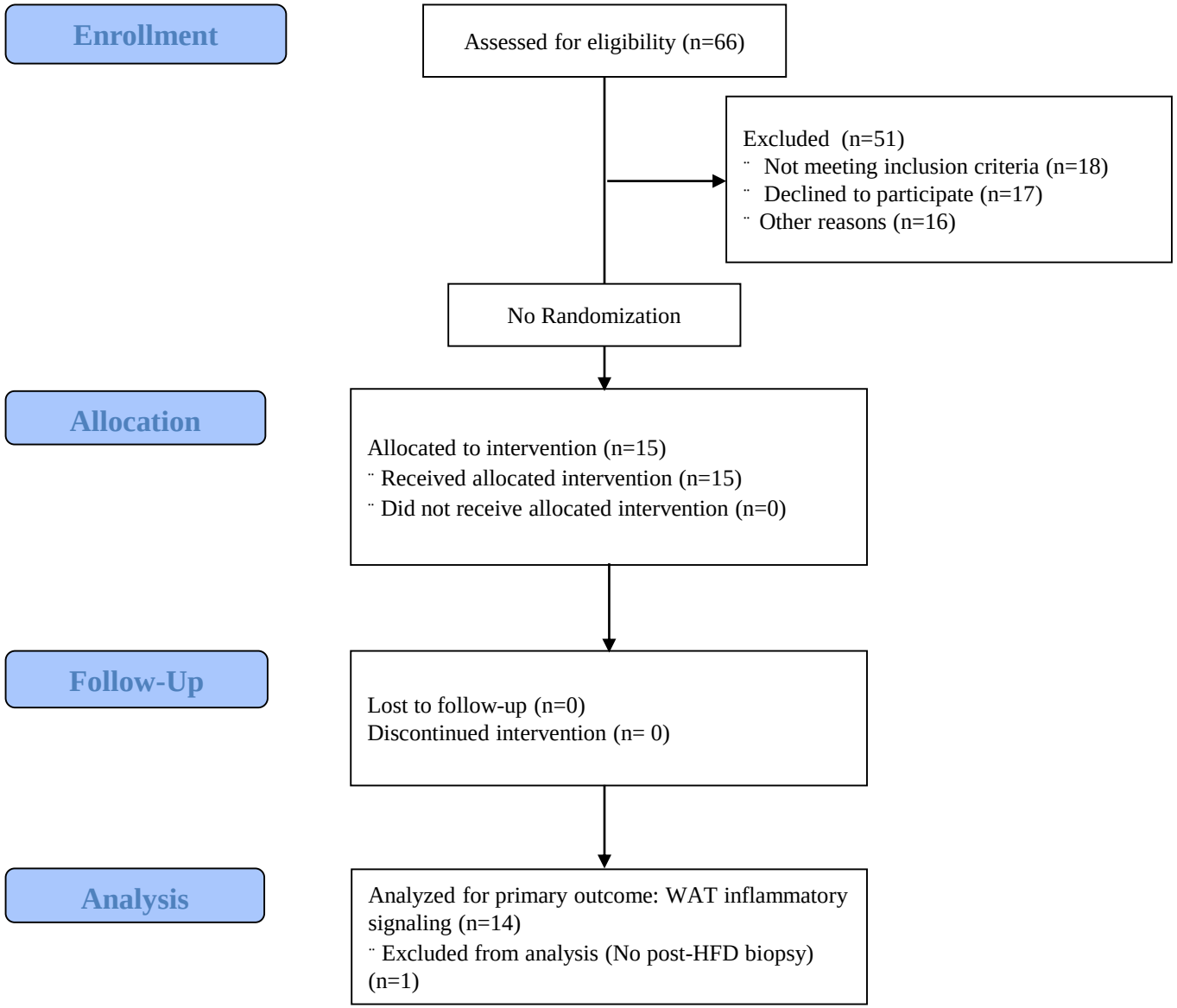

Figure 3

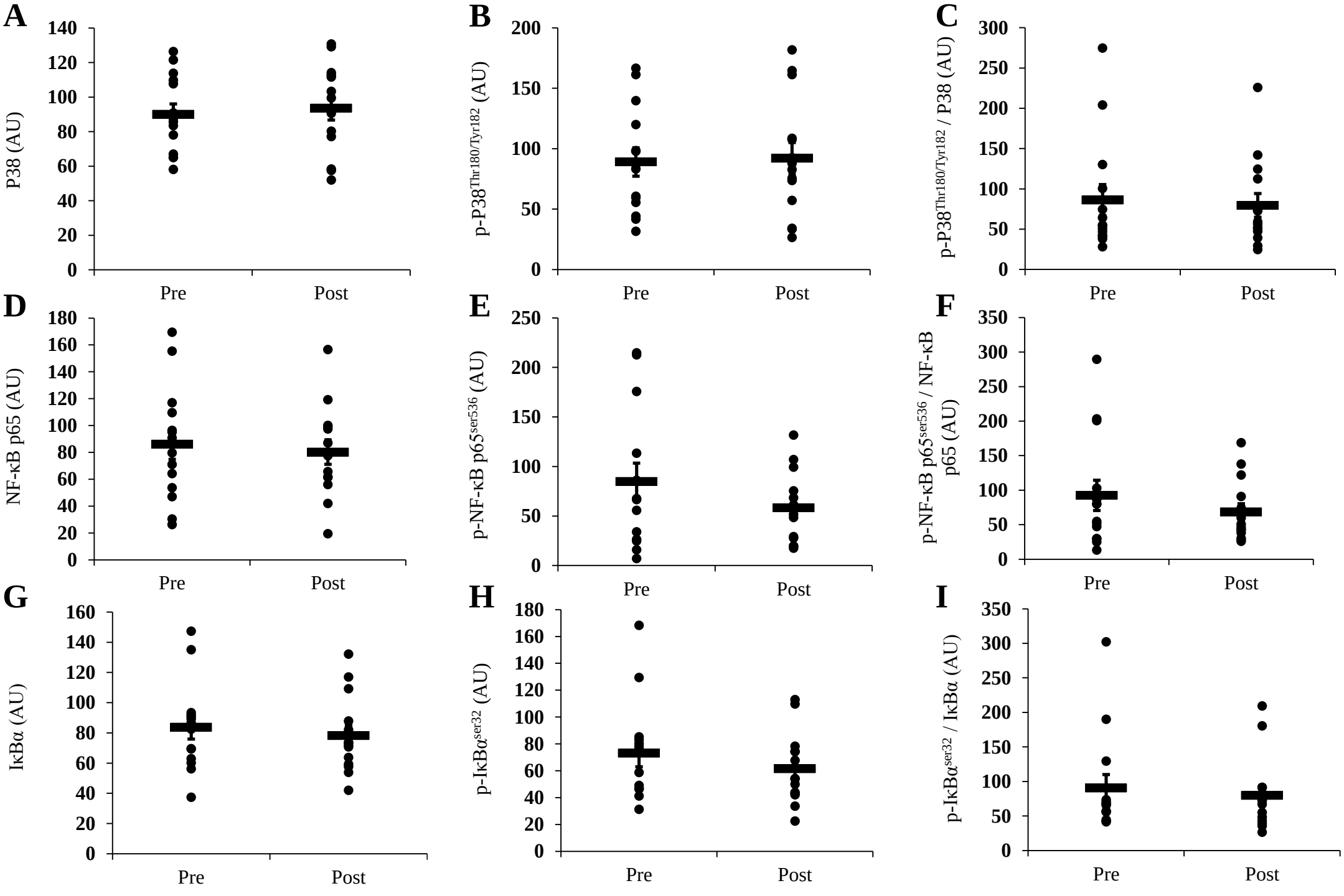

Figure 4

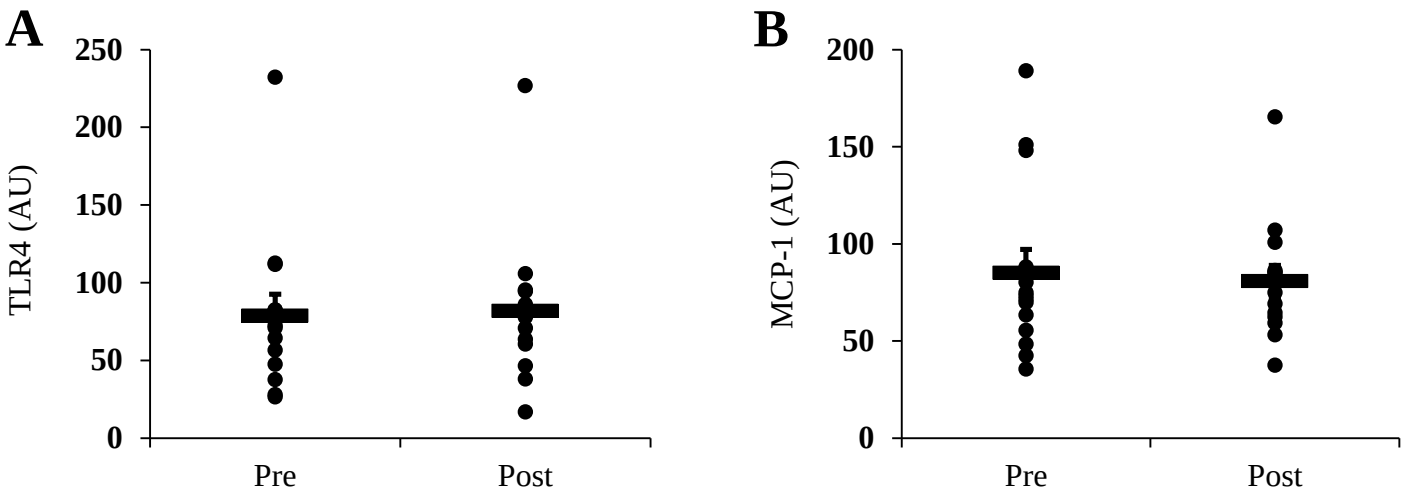

Figure 5