ACCEPTED MANUSCRIPT

Weight loss achieved using an energy restriction diet with normal or higher dietary protein decreased the number of CD14^{++}CD16^{+} pro-inflammatory monocytes and plasma lipids and lipoproteins in middle-aged, overweight and obese adults

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List of Abbreviations (alphabetical order):

HP: high protein

MPFC: multi-parametric flow cytometry

MNCs: mononuclear cells

MS: monocyte subpopulations

NP: normal protein

TC: total-cholesterol

TG: triglyceride

HDL: high density lipoprotein cholesterol

LDL: low density lipoprotein cholesterol

UUC: urinary urea nitrogen

Abstract:

Monocytes are involved in immune responses and specific monocyte subpopulations (MS) that express intermediate to high levels of CD16 are associated with obesity and cardiovascular events. Consuming high protein (HP) when dieting improves body composition and cardio-metabolic health outcomes, but whether HP affects MS during weight loss remains unknown. We assessed the effect of HP on energy restriction (ER)-induced changes in MS in overweight and obese adults. The relations between MS and plasma lipids and lipoproteins were also examined. We hypothesized that independent of protein intake, ER-induced weight loss would decrease the numbers of MS and that MS and plasma lipids and lipoproteins would be related. Thirty-two adults (age 52 ± 1y, BMI 31.3 ± 0.5kg/m^2, means$\pm$SE) consumed either a normal protein (n=18) or HP (n=14) (0.8 vs. 1.5g•kg^{-1}•d^{-1} protein) ER diet (750kcal/d deficit) for 16 wk. The HP diet included 0.7g•kg^{-1}•d^{-1} of milk protein isolate. Fasting plasma lipids, lipoproteins, and the numbers of MS were analyzed. Over time, independent of protein intake, CD14^{++}CD16^{+} cell number decreased, while CD14^{dim}CD16^{++}, CD14^{+}CD16^{+}, and CD14^{+}CD16^{-} cell numbers remained unchanged. CD14^{dim}CD16^{++} cell number was negatively associated with total-cholesterol (TC) and triglyceride, while CD14^{++}CD16^{+} cell number was positively associated with TC, low-density lipoprotein cholesterol (LDL), TC to high density lipoprotein cholesterol (HDL) ratio, and LDL to HDL ratio. Weight loss achieved while consuming an ER diet with either normal or high protein may improve immunity by partially decreasing pro-inflammatory monocytes. Associations between MS and plasma lipids and lipoproteins are confirmed in overweight and obese adults.

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1. Introduction

Obesity is associated with an increased risk of cardio-metabolic diseases including cardiovascular disease and type 2 diabetes [1-3]. It is well known that obesity induces unfavorable blood plasma lipid and lipoprotein profiles [3] and activation of innate immunity [4, 5], resulting in the development of atherosclerosis and insulin resistance. In particular, monocytes are involved with the regulation of innate immunity, including phagocytosis, secretion of inflammatory cytokines, and production of reactive oxygen species [6, 7]. Therefore, there is an emerging research interest to better understand how modulating circulating monocytes may impact obesity-induced cardio-metabolic diseases.

Based on the different levels of expression of CD14 and CD16 cell-surface markers [8], human blood monocytes display heterogeneous subpopulations [9, 10]. Particularly, monocytes that express intermediate to high levels of CD16, also known as CD16^{+} monocytes, are considered pro-inflammatory monocytes [11, 12], and research implicates these monocytes in obesity [6, 13], inflammatory conditions including atherosclerosis [13, 14], and cardiovascular events and endpoint (cardiovascular death, acute myocardial infarction or non-hemorrhagic stroke) [15, 16].

Purposeful weight loss is an effective way for adults with excess adiposity to improve body composition and cardio-metabolic health outcomes [1, 17]. Importantly, consumption of higher amounts of protein while dieting promotes greater body fat loss and fat-free mass retention, which are considered positive outcomes for health [1, 18]. However, the impact of higher dietary protein intake on changes in the various monocyte subpopulations (MS) during weight loss remains unknown.

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The purpose of this study was to assess the effect of a higher dietary protein intake on energy restriction-induced changes in MS and plasma lipids and lipoproteins in overweight and obese adults. This assessment was a secondary analysis of data from a study designed to investigate the effect of higher-protein intake on cardiovascular disease and diabetes risk factors and body composition after weight loss. Our first specific aim was to investigate the effect of higher dietary protein on changes in MS while consuming an energy restriction (ER) diet. We hypothesized that independent of protein intake ER-induced weight loss would decrease the cell numbers of the various MS. Our second specific aim was to examine the relations between the various MS and plasma lipids and lipoproteins. We hypothesized that the different MS and plasma lipids and lipoproteins would be associated with each other. Experimentally, these hypotheses were tested by measuring the cell numbers of the blood MS and plasma lipids and lipoproteins concentrations before and after these subjects completed a 16-wk controlled feeding intervention.

2. Methods and Materials

2.1 Subjects

Sixty-nine overweight and obese adults (body mass index (BMI) range: 25-38 kg/m^2) were recruited from the greater Lafayette, Indiana, region, and 48 of 69 subjects completed the original intervention (9 discontinued the baseline and 12 discontinued the intervention). Among the 48 subjects, data on the MS were collected from 32 subjects and analyzed for this research (4 were deemed noncompliant with the original intervention; blood collected from 11 were not able to be analyzed due to issues with blood collection and shipping; and 1 provided an abnormal white blood cell number) (Figure 1). Inclusion criteria for this study were as follows; either male or female; aged 35-65 y; weight stable (±3 kg) during last 3 months; no acute illness; not diabetic, pregnant or lactating; not currently (or within last 3 months) following an exercise or weight loss program; non-smoking; not lactose intolerant; natural waist circumference ≥102 cm for male and ≥88 cm for female), blood pressure <140/90 mmHg; fasting serum glucose concentration <110 mg/dL; fasting serum total-cholesterol (TC) concentration <260 mg/dL; fasting serum low density lipoprotein cholesterol (LDL) concentration <160 mg/dL; fasting serum triglyceride (TG) concentration <400 mg/dL; and normal albumin and pre-albumin concentrations. All testing procedures were approved by the Purdue University Biomedical Institutional Review Board. Each subject signed an informed consent form and a monetary stipend was provided for participation. This trial was registered at clinicaltrials.gov as NCT01692860.

2.2 Experimental Design

This experiment was a 20-wk randomized, parallel, placebo controlled, double blind, and prospective study (1-wk pre study measurement, 3-wk controlled feeding baseline, and 16-wk controlled feeding, ER intervention) design. After the 3-wk baseline period, subjects were randomly allocated to either a high protein (HP, n=14) or normal protein (NP, n=18) diet and consumed an ER diet for 16 wk. Randomization was conducted using the first generator (6 participants/block with separate random assignments; 10 blocks for women and 4 blocks for men) on Randomization.com.

2.3 Diet Intervention

Total energy requirement for each subject was estimated using sex-specific equations for overweight and obese adults [19]. During the 3-wk baseline period, all subjects consumed an energy-balance diet providing 0.8 g protein•kg body mass^{-1}•d^{-1} and selected food and beverage items contained 0.7 g•kg body mass^{-1}•d^{-1} of the carbohydrate powder maltodextrin (Muscle Feast LLC, Hebron, OH). During the 16-wk intervention period, subjects were randomly assigned to either the NP group (total protein intake 0.8 g protein•kg body mass^{-1}•d^{-1}) or HP group (total protein intake 1.5 g protein•kg body mass^{-1}•d^{-1}). Subjects in the NP group continued to consume selected food and beverage items containing maltodextrin, while subjects in the HP group switched to the consumption of these food and beverage items containing 0.7 g protein•kg body mass^{-1}•d^{-1} of milk protein isolate-85 (MPI-85) (Idaho Milk Products, Inc. Jerome, ID). The ER (a 750 kcal energy deficit per day) was achieved by excluding non-protein foods and beverages from each subject's energy-balance diet menu. All subjects self-purchased most foods and beverages based on counseled 7-d rotating

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menus and shopping lists, and were provided the selected foods and beverages that contained the maltodextrin or MPI-85 powders. Twenty-four-hour urinary urea nitrogen (UUN)/Creatinine ratio was assessed at wk 1, 4, 8, 12, 16, and 20 as an indicator of compliance to the diet, consistent with differences in total protein intake.

2.4 Body Composition

Fasting-state body mass, adjusted for clothing mass, was measured (±0.01 kg) using a digital platform scale (model ES200L, Ohaus Corporation, Pine Brook, NJ). Height (±0.1 cm) was measured from a standing body without shoes using a wall-mounted stadiometer. BMI (kg/m^2) was calculated from the collected body mass and height measurements. Fasting-state body mass was also measured weekly during baseline and the intervention period to document compliance to the ER diet.

2.5 White Blood Cells, Plasma Lipids and Lipoproteins Analyses

Fasting state blood samples were collected at the end of baseline week 3 and intervention week 16. Subjects arrived at the Purdue Clinical Research Center in the morning. Subjects then rested in a seated position for 15 min and fasting blood samples were drawn. Whole blood samples were aliquoted into EDTA tubes (BD Biosciences) and sent to Mid America Clinical Laboratory (Indianapolis, IN) for the evaluation of white blood cells, neutrophils, lymphocytes, monocytes, and eosinophils using flow-cytometric methodology and an ADVIA 2120i Hematology system (Siemens Medical Solutions USA, Inc., Malvern, PA). Blood samples were also aliquoted into EDTA tubes (BD Biosciences) and centrifuged at 4°C for 10 min at 3000 × g, and aliquots of plasma were

stored at −80°C until thawed for lipids and lipoproteins analyses. Plasma TC, high density lipoprotein cholesterol (HDL), and TG concentrations were measured in duplicate using an oxidase method on a COBAS analyzer (Roche Diagnostic Systems, Indianapolis, IN), and LDL was estimated using the Friedewald equation [20]. TC/HDL and LDL/HDL ratios were calculated by dividing TC and LDL by HDL, respectively.

Additionally, 8mL of whole blood was collected into cell preparation tubes (CPT, BD Biosciences), with the mononuclear cells (MNCs) being isolated following a centrifugation at 1,600g for 30 minutes at room temperature. The MNC fraction was sent to the Angio BioCore located at Indiana University, Indianapolis, IN, for the enumeration of four MS using a 7-color multi-parametric flow cytometry (MPFC) protocol as previously described [21-23]. To ensure reproducibility, the MNCs were stained and fixed within 24 hours after initial blood collection, with fixed samples being run on the cytometer within 72 hours.

2.6 MPFC Immunostaining, Acquisition and Analysis

The following primary conjugated monoclonal antibodies were used: anti-human CD31 fluoroscein isothyocyanate (FITC, BD Pharmingen, cat. no. 555445), anti-human CD34 phycoerythrin (PE, BD Pharmingen, cat. no. 550761), anti-human AC133 allophycocyanin (APC, Miltenyi Biotec cat. no. 130-090-826), anti-human CD14 PECy5.5 (Abcam, cat. no. ab25395), anti-human CD45 APC-AlexaFluor (AF) 750 (Invitrogen, cat. no. MHCD4527), anti-human CD16 PECy7 (BD Pharmingen, cat. no. 557744), and the fixable amine reactive viability dye, LiveDead (Violet, Invitrogen cat. no. L34955). In order to resolve the cell populations of interest, specific antigen and

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fluorochrome conjugate coupling was optimized for the six-antibody plus viability marker

staining panel [21-23]. Both endothelial and hematopoietic stem/progenitor cells were

evaluated as previously published [21-23]. The four MS evaluated were

$CD14^{dim}CD16^{++}$, $CD14^{+}CD16^{+}$, $CD14^{++}CD16^{+}$, and $CD14^{++}CD16^{-}$ cells.

Blood MNCs were incubated with a proprietary Fc blocking reagent (Miltenyi Biotec

Inc.) for 10 minutes on ice and stained as previously described [21-23]. Briefly, cells

were incubated with titered antibodies for 30 minutes at 4°C, washed twice in PBS with

2% fetal bovine serum, fixed in 1% paraformaldehyde (Tousimis, Rockville, MD), and

subsequently run on a BD LSRII flow cytometer (BD, Franklin Lakes, NJ) equipped with

a 405nm violet laser, 488nm blue laser and 633nm red laser. Data were acquired

uncompensated and exported as FCS 3.0 files, and analyzed utilizing FlowJo software,

version 9.7.6 (Tree Star, Inc). "Fluorescent minus one" gating controls were also used to

ensure proper gating of positive events [21, 22].

The monocytes were expressed as a percentage of the total circulating MNC

population obtained from the MPFC analysis (Figure 2.). Absolute values of each MS

were then calculated using the absolute monocyte numbers from the complete blood

number results analyzed by Mid America Clinical Laboratory.

2.7 Power Calculation and Statistical Analyses

Since the assessment of changes in MS was a secondary objective of the original

study, subject sample size estimates were not done based on this outcome of interest.

Retrospectively, we conducted an effect-size calculation. For this parallel designed

study, a power calculation was completed for 2 independent means to detect a

difference equal to 1 SD between HP and NP (a=0.05; 80% power; 2 tailed). The effect size was one, and the number of participants needed was 34 (n=17/group). Our sample size (n=32) offered us 78% actual power.

Independent t-tests were applied to assess the differences between HP and NP groups for baseline subject characteristics and 24 hour UUN/Creatinine ratio. Repeated measures ANOVA (MIXED Procedure) were used to assess the main effects of time, diet, and time-by-diet interaction using age and gender as covariates. Paired t-tests were also used to compare differences between baseline and post-intervention. A multiple linear regression model was used to assess associations between the monocyte numbers and concentrations of plasma lipids and lipoproteins, with all estimates adjusted for age and gender. All statistical analyses were completed using SAS Version 9.2 (SAS Institute Inc. Cary, NC) and data are presented as least-square means±SE, unless otherwise noted. Statistical significance was determined at $p<0.05$ for all analyses.

3. Results

3.1 Participants' Baseline Characteristics

The means±SE of age, body mass, and BMI in HP (4 males and 10 females) and NP (5 males and 13 females) groups were as follows: 51±1.5 y, 89.3±2.8 kg, and 31.6±1.0 kg/m^2 and 52±2.0 y, 84.3±2.6 kg, and 31.1±0.6 kg/m^2, respectively. No statistical differences were observed in these variables between the HP and NP groups.

3.2 Dietary Compliance

All subjects were compliant with the ER diet on the basis of decreased body mass and BMI after the 16-wk intervention period (Table 1) and gradual declines of weekly body mass (Figure 3). No differences were observed for 24 hour UUN/Creatinine ratio between HP and NP at baseline week 3, however, it was higher in HP than NP during the 16-wk intervention period (Figure 4).

3.3 Changes in Plasma Lipids, Lipoproteins, and White Blood Cell Numbers

From baseline week 3 to intervention week 16 (i.e. over time), TG, TC, LDL, TC/HDL ratio, and LDL/HDL ratio were all decreased, whereas HDL was increased in both HP and NP (Figure 5 and Table 1). Total numbers of white blood cells, neutrophils, lymphocytes, monocytes, and eosinophils remained unchanged over time (Table 1).

3.4 Changes in Monocyte Numbers

No change was observed for any of the endothelial or hematopoietic stem/progenitor cell populations (data not shown). However, protein intake did change the specific

numbers of MS throughout the study. Over time, $CD14^{++}CD16^{+}$ cell numbers decreased, while $CD14^{dim}CD16^{++}$, $CD14^{+}CD16^{+}$, and $CD14^{+}CD16^{-}$ cell numbers remained unchanged, independent of protein intake (Figure 6 and Table 2).

3.5 Relationship between Monocyte Numbers and Plasma Lipids and Lipoproteins Profiles

Table 3 presents associations between the monocyte numbers and lipids or lipoproteins using data from both baseline and post-intervention. Expression of $CD14^{dim}CD16^{++}$ cells was shown to be negatively associated with TC and TG, but not with other variables. $CD14^{+}CD16^{+}$ cell expression was not associated with lipids and lipoproteins, whereas $CD14^{++}CD16^{+}$ cells were shown to be positively associated with TC, LDL, TC/HDL ratio, and LDL/HDL ratio. Additionally, no associations were observed between $CD14^{++}CD16^{-}$ cells and lipids or lipoproteins. Over time, changes in the monocyte numbers were not associated with changes in lipids and lipoproteins (Table 4).

4. Discussion

Monocytes are involved in obesity-induced immune response [4-6], and mounting evidence suggests that specific MS that express intermediate to high levels of CD16 (CD16$^+$ and CD16^{++}) are associated with obesity [6, 13] and cardiovascular events [15, 16]. Very limited research has assessed changes in MS during weight loss [6, 24]. Consistent with our hypothesis, ER-induced weight loss improved CD14^{++}CD16$^+$ monocyte numbers and lipids and lipoproteins independent of dietary protein intake in overweight and obese adults. Our findings also confirm positive relations between CD14^{++}CD16$^+$ monocyte numbers and plasma lipids and lipoproteins.

Weight loss may improve obesity-induced immune response by decreasing the monocyte numbers and pro-inflammatory cytokines [4]. Although findings from human intervention studies are limited, some studies particularly investigated the impact of various types of weight loss on changes in MS. One study observed a reduction of CD14$^+$CD16$^+$ cells after surgery-induced weight loss in obese subjects [6]. However, consistent with our findings, no changes in CD14$^+$CD16$^+$ cells were observed after 6 [6] to 12 [24] weeks of ER-induced weight loss. We did however observe a reduction in the number of CD14^{++}CD16$^+$ cells (known as intermediate or pro-inflammatory monocytes) [11, 12] after 16 weeks of ER-induced weight loss. This result may suggest that ER-induced weight loss contributes to improving immunity by decreasing this distinct subpopulation of monocyte.

While beneficial effects of HP intake on body composition and cardio-metabolic health during weight loss are well defined [18, 25], its impact on immune response, particularly during weight loss in overweight and obese adults, is unclear. Limited

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animal research suggests sulfur-containing amino acids, methionine and cysteine, modulate immune responses [26]. In humans, the impact of higher dietary protein intake achieved by consuming either beef or chicken on innate immunity was assessed in obese postmenopausal women after nine weeks of ER-induced weight loss [27]. HP intake did not affect the majority of indices of innate immunity, including white blood cell numbers, consistent with our current findings.

Interactions between MS and lipid or lipoprotein metabolism were reported in several *in vitro* studies [28, 29]. Multiple human clinical studies also observed associations between MS, CD16[+/++] in particular, and blood lipid and lipoprotein concentrations [6, 13, 30-32]. CD16^{+} monocytes were positively correlated with TC, LDL, and TG concentrations in hypercholesterolemic patients with coronary heart disease [30] and in lean to obese adults [6]. In contrast, CD16^{+} monocytes were negatively correlated with HDL concentration in lean to obese [6] and healthy [13] adults. In our study, we also found positive correlations between CD16^{+} monocytes number and lipid and lipoprotein concentrations in middle-aged overweight and obese adults. Collectively, higher cell numbers for CD16[+/++] monocytes may contribute to worsened plasma lipids and lipoproteins profiles. However, these associations need to be interpreted with caution because they are not cause and effect relations.

The novelty of this study is supported by the lack of human intervention studies assessing the impact of higher dietary protein intake on changes in MS during weight loss in overweight and obese adults applying a randomized controlled study design. However, we recognize that MS were analyzed from only 32 of 44 participants who completed the original intervention, mostly due to unexpected difficulties with blood

sample collection and processing. Another limitation may be that other immune-related cells and immune function may contribute to immune response. Thus, more research is needed to assess the impact of ER-induced weight loss and dietary protein intake on those components.

Our experimental design included measuring outcomes of interest at baseline while subjects consumed an energy balance diet while the post-intervention samples were obtained while subjects consumed an ER diet. Thus, our experimental design does not allow us to distinguish between the effects of weight loss versus dietary ER on changes in the MS. This issue is pertinent to comparable research studies.

In summary, ER-induced weight loss may improve immunity by partially decreasing the cells number of pro-inflammatory monocytes (CD14^{++}CD16^{+} cell), in middle-aged, overweight and obese adults who consumed either normal or higher amounts of dietary protein. Distribution of distinct MS may also predict plasma concentrations of lipids and lipoproteins in these adults at risk for cardiovascular disease.

Acknowledgements

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Table 1. Plasma lipids, lipoproteins, and white blood cell counts

	HP pre	HP post	NP pre	NP post	Time effect, p	Group effect, p	Time-by-group interaction, p
Body mass (kg)	89.4 ± 2.5	80.5 ± 2.5	84.5 ± 2.2	76.5 ± 2.2	<0.0001	0.16	0.41
BMI (kg/m^2)	30.8 ± 0.8	27.6 ± 0.8	30.0 ± 0.7	27.0 ± 0.7	<0.0001	0.48	0.70
Lipids and lipoproteins							
TC (mg/dL)	173.5 ± 7.2	148.1 ± 7.2	176.2 ± 6.4	159.7 ± 6.4	<0.0001	0.42	0.18
TG (mg/dL)	136.2 ± 11.5	81.7 ± 11.5	120.7 ± 10.3	86.7 ± 10.3	<0.0001	0.67	0.23
HDL (mg/dL)	41.7 ± 3.1	44.7 ± 3.1	44.4 ± 2.7	47.9 ± 2.7	0.004	0.44	0.77
LDL (mg/dL)	104.5 ± 7.0	86.8 ± 7.0	107.5 ± 6.2	94.3 ± 6.2	<0.0001	0.54	0.43
TC/HDL	4.4 ± 0.3	3.4 ± 0.3	4.2 ± 0.2	3.5 ± 0.2	<0.0001	0.86	0.14
LDL/HDL	2.7 ± 0.2	2.0 ± 0.2	2.6 ± 0.2	2.1 ± 0.2	<0.0001	0.98	0.27
White blood cell counts							
White blood cells (10^6/mL)	5.83 ± 0.41	5.77 ± 0.41	6.01 ± 0.37	5.74 ± 0.37	0.29	0.88	0.50
Neutrophils (10^6/mL)	3.38 ± 0.35	3.34 ± 0.35	3.52 ± 0.31	3.47 ± 0.31	0.72	0.76	0.98
Lymphocytes (10^6/mL)	1.86 ± 0.12	1.81 ± 0.12	1.91 ± 0.11	1.70 ± 0.11	0.09	0.82	0.32
Monocytes (10^6/mL)	0.42 ± 0.03	0.43 ±0.03	0.39 ± 0.03	0.39 ± 0.03	0.84	0.32	0.84
Eosinophils (10^6/mL)	0.12 ± 0.03	0.17 ± 0.03	0.15 ± 0.03	0.16 ± 0.03	0.08	0.65	0.26

Results are reported as lsmeans±SE. Analyses are adjusted for age and gender. HP, high protein; NP, normal protein; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol.

Table 2. Monocyte subpopulation cell numbers

	HP pre	HP post	NP pre	NP post	Time effect, p	Group effect, p	Time-by-group interaction, p
CD14^{dim}CD16^{++} (10^6/mL)	0.0003 ± 0.009	0.0003 ± 0.009	0.0009 ± 0.0008	0.0026 ± 0.0008	0.30	0.09	0.28
CD14$^+$CD16$^+$ (10^6/mL)	0.023 ± 0.005	0.028 ± 0.005	0.023 ± 0.004	0.031 ± 0.004	0.18	0.73	0.72
CD14^{++}CD16$^+$ (10^6/mL)	0.016 ± 0.003	0.009 ± 0.003	0.013 ± 0.002	0.005 ± 0.002	0.0005	0.21	0.96
CD14^{++}CD16$^-$ (10^6/mL)	0.321 ± 0.030	0.346 ± 0.030	0.296 ± 0.027	0.291 ± 0.027	0.56	0.25	0.37

Results are reported as lsmeans±SE. Analyses are adjusted for age and gender. HP, high protein; NP, normal protein.

Table 3. Associations between monocytes subpopulations and lipids and lipoproteins

	$CD14^{dim}CD16^{++}$ (10^6/mL)		$CD14^+CD16^+$ (10^6/mL)		$CD14^{++}CD16^+$ (10^6/mL)		$CD14^{++}CD16^-$ (10^6/mL)	
	β* (95% CI)	p	β* (95% CI)	p	β* (95% CI)	p	β* (95% CI)	p
TC (mg/dL)	-2153 (-4293, -14)	0.049	83 (-309, 475)	0.67	1087 (437, 1737)	0.001	30 (-37, 96)	0.38
TG (mg/dL)	-3561 (-7189, 67)	0.05	-72 (-736, 593)	0.83	607 (-582, 1796)	0.31	102 (-9, 213)	0.07
HDL (mg/dL)	126 (-744, 996)	0.77	130 (-21, 281)	0.09	-210 (-484, 64)	0.13	-15 (-42, 11)	0.24
LDL (mg/dL)	-1546 (-3551, 458)	0.13	-41 (-404, 322)	0.82	1179 (600, 1759)	< 0.001	24 (-38, 86)	0.44
TC/HDL	-67 (-147, 14)	0.11	-7 (-21, 8)	0.36	41 (16, 65)	0.001	2 (-1, 4)	0.20
LDL/HDL	-45 (-109, 19)	0.16	-6 (-17, 6)	0.32	37 (18, 55)	< 0.001	1 (-1, 3)	0.28

* Estimates of adjusted regression coefficient between monocytes and lipids and lipoproteins. All estimates are adjusted for age and gender. TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol.

Table 4. Associations between changes in monocytes subpopulations and changes in lipids and lipoproteins

	Δ CD14^{dim}CD16^{++} (10^6/mL)		Δ CD14$^+$CD16$^+$ (10^6/mL)		Δ CD14^{++}CD16$^+$ (10^6/mL)		Δ CD14^{++}CD16$^-$ (10^6/mL)	
	β* (95% CI)	p	β* (95% CI)	p	β* (95% CI)	p	β* (95% CI)	p
Δ TC (mg/dL)	-403 (-1980, 1174)	0.61	231 (-19, 482)	0.07	-202 (-864, 460)	0.54	7 (-73, 87)	0.86
Δ TG (mg/dL)	606 (-2955, 4167)	0.73	280 (-309, 869)	0.34	-222 (-1754, 1311)	0.77	60 (-123, 242)	0.51
Δ HDL (mg/dL)	-178 (-523, 167)	0.30	36 (-21, 94)	0.21	-70 (-220, 79)	0.35	-12 (-29, 6)	0.19
Δ LDL (mg/dL)	-312 (-1609, 985)	0.63	134 (-79, 346)	0.21	-72 (-623, 479)	0.79	6 (-61, 72)	0.86
Δ TC/HDL	8 (-42, 58)	0.74	4 (-4, 12)	0.31	6 (-15, 26)	0.59	1 (-2, 3)	0.47
Δ LDL/HDL	4 (-32, 41)	0.81	3 (-4, 9)	0.41	6 (-10, 21)	0.46	1 (-1, 2)	0.54

* Estimates of adjusted regression coefficient between monocytes with lipid-lipoproteins. All estimates are adjusted for age and gender. TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol.

Figure 1. Consort flow diagram

Enrollment

Assessed for eligibility
(n=114; 29M:85F)

Excluded (not meeting inclusion
criteria or declined to participate)
(n=45; 13M:32F)

Entered study (n=69; 16M:53F)

Discontinued baseline
(Underestimated the
commitment to the study,
personal reason, medical
issues, or not willing to
consume the study diet)
(n=9; 1M:8F)

Completed baseline
(n=60; 15M:45F)

Allocation

Allocated to intervention
HP (n=29; 7M:22F)

Allocated to intervention
NP (n=31: 8M:23F)

Discontinued
intervention (Non-
compliance or personal
reason) (n=6 (21%);
2M:4F)

Discontinued intervention
(Non-compliance,
personal reason, or
change of medication for
blood pressure) (n=6
(19%); 1M:5F)

Completed study
(n=23; 5M:18F)

Completed study
(n=25; 7M:18F)

Excluded from analyses
(did not lose weight or
failed to comply to the
assigned protein intake)
(n=2; 2F)

Excluded from analyses
(did not lose weight or
failed to comply to the
assigned protein intake)
(n=2; 2F)

Analysis

Analyzed (n=21; 5M:16F)

Analyzed (n=23; 7M:16F)

Excluded from analyses
(missing blood sample
or cell death during
shipping) (n=6; 1M:5F)

Excluded from analyses
(missing blood sample
or cell death during
shipping) (n=5; 2M:3F)

Analyzed (n=15; 4M:11F)

Excluded from analyses
(abnormal white blood
cell count) (n=1; 1F)

Analyzed (n=18; 5M:13F)
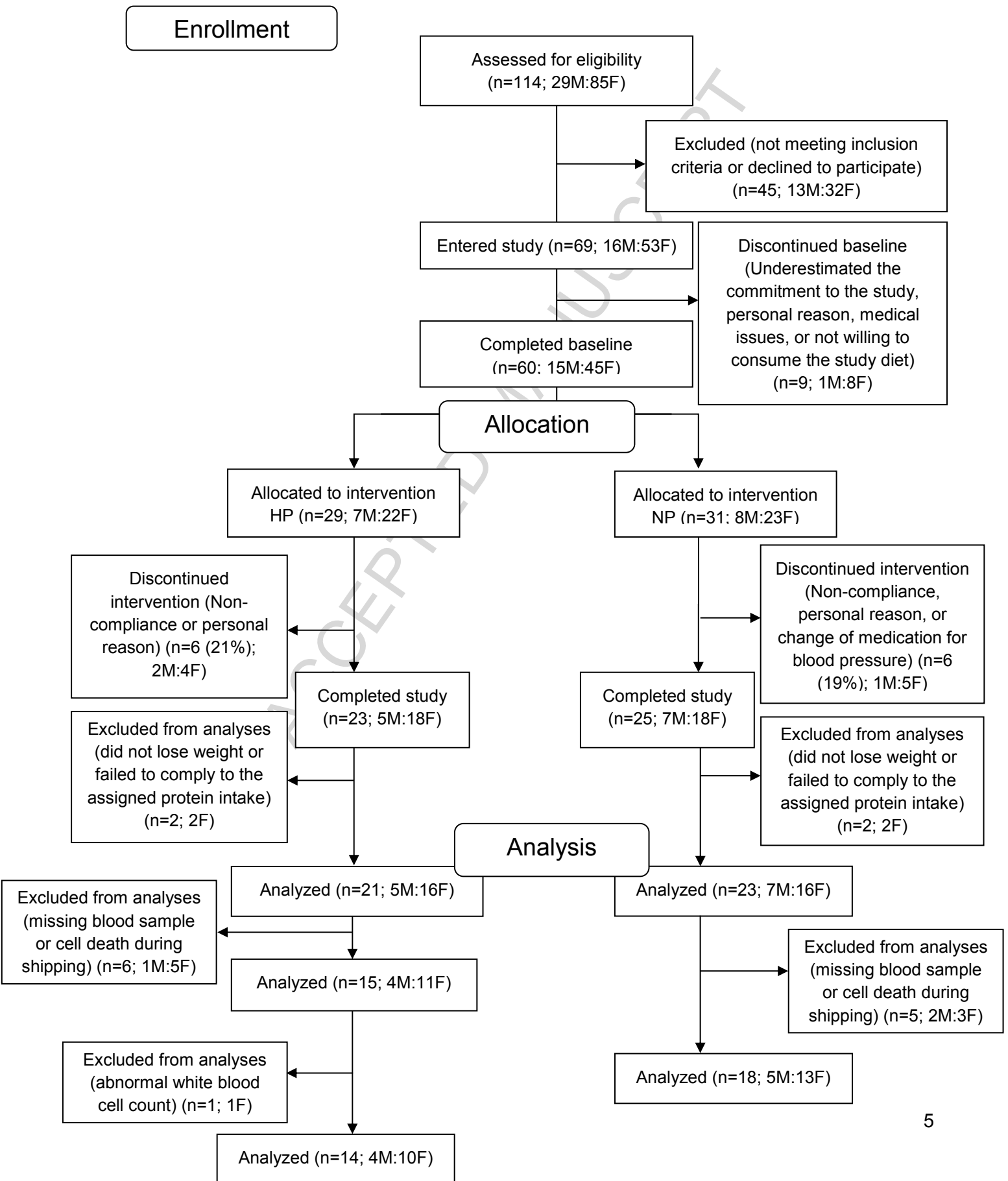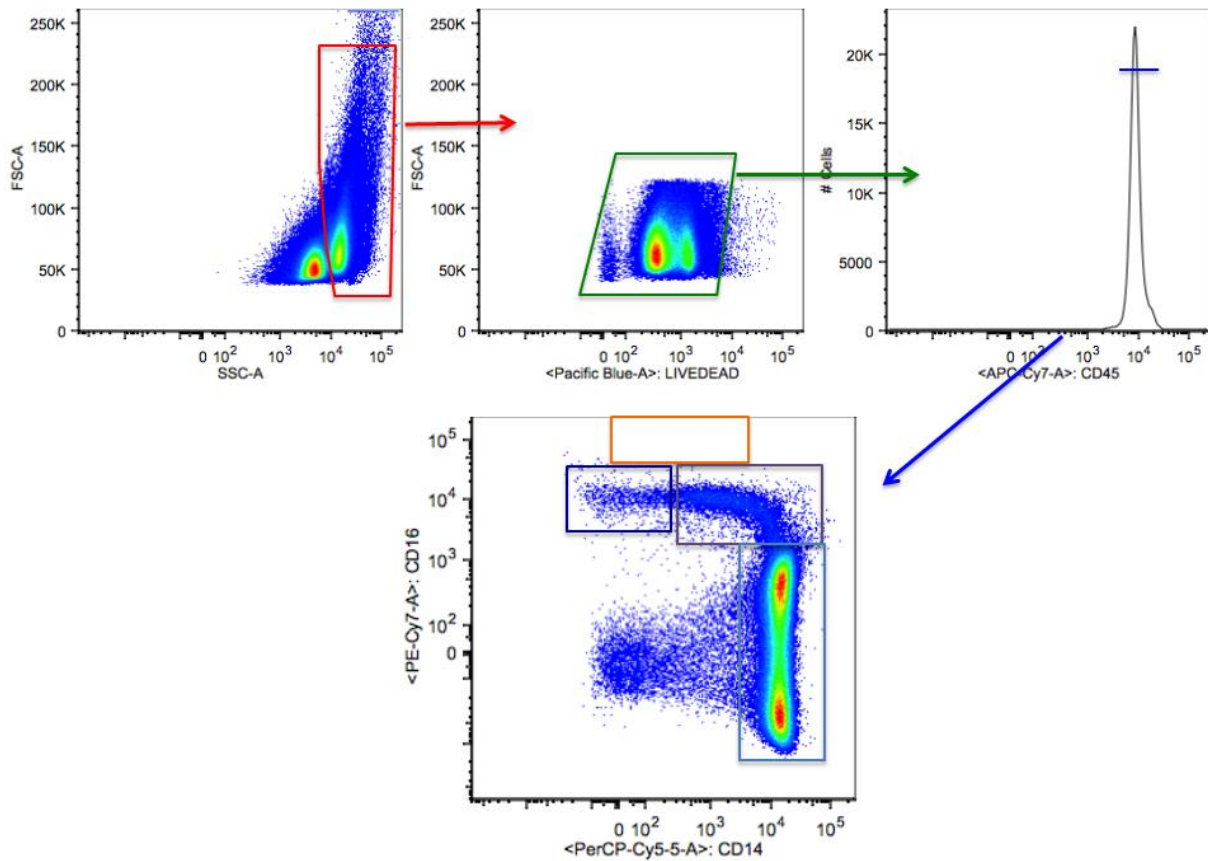
Analyzed (n=14; 4M:10F)

5

**Figure 2. Representative gating strategy of human monocyte populations based on CD14 and CD16 expression**

High SSC monocytes (red box) are gated into a LIVEDEAD plot (green box). As monocytes have a higher autofluoresence, live cells are shifted to the positive decades. Live cells are gated for CD45 through histogram into the four monocyte populations. CD14dimCD16++ (orange box), CD14-CD16+ (dark blue box), CD14+CD16+ (purple box), and CD14+CD16- (light blue box).

**Figure 3. Change in body mass during the 16-wk intervention period**
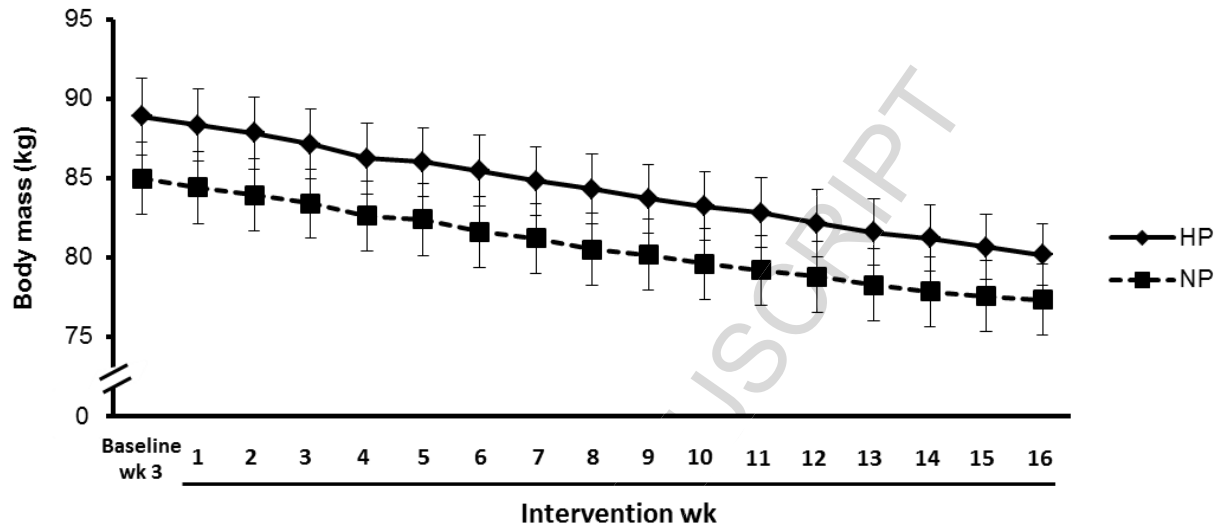
Results are reported as means±SE. Statistical main effect of time, $p<0.0001$; main effect of group, $p=0.14$; time-by-group interaction, $p=0.78$. HP, high protein; NP, normal protein.

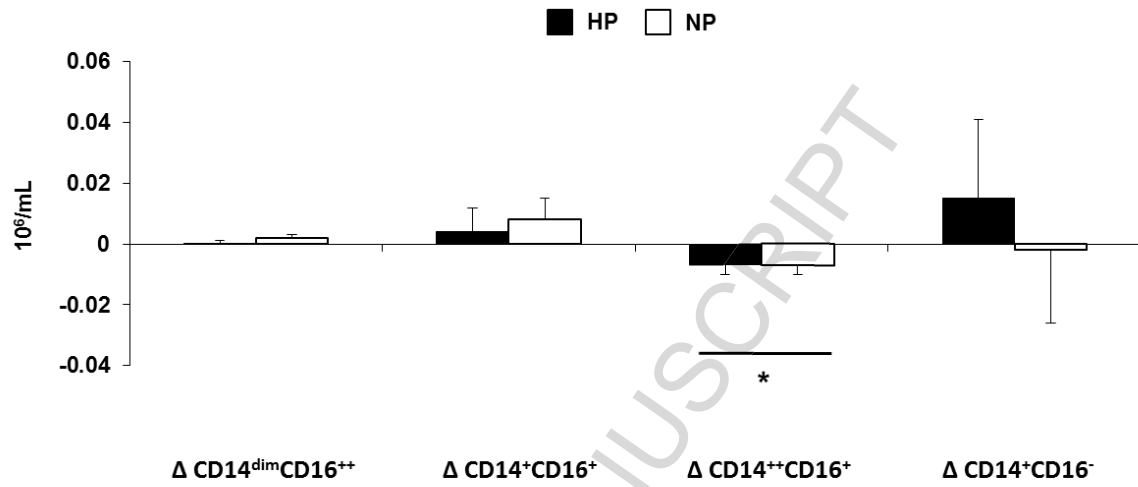**Figure 4. Change in 24 hour UUN/Creatinine ratio**
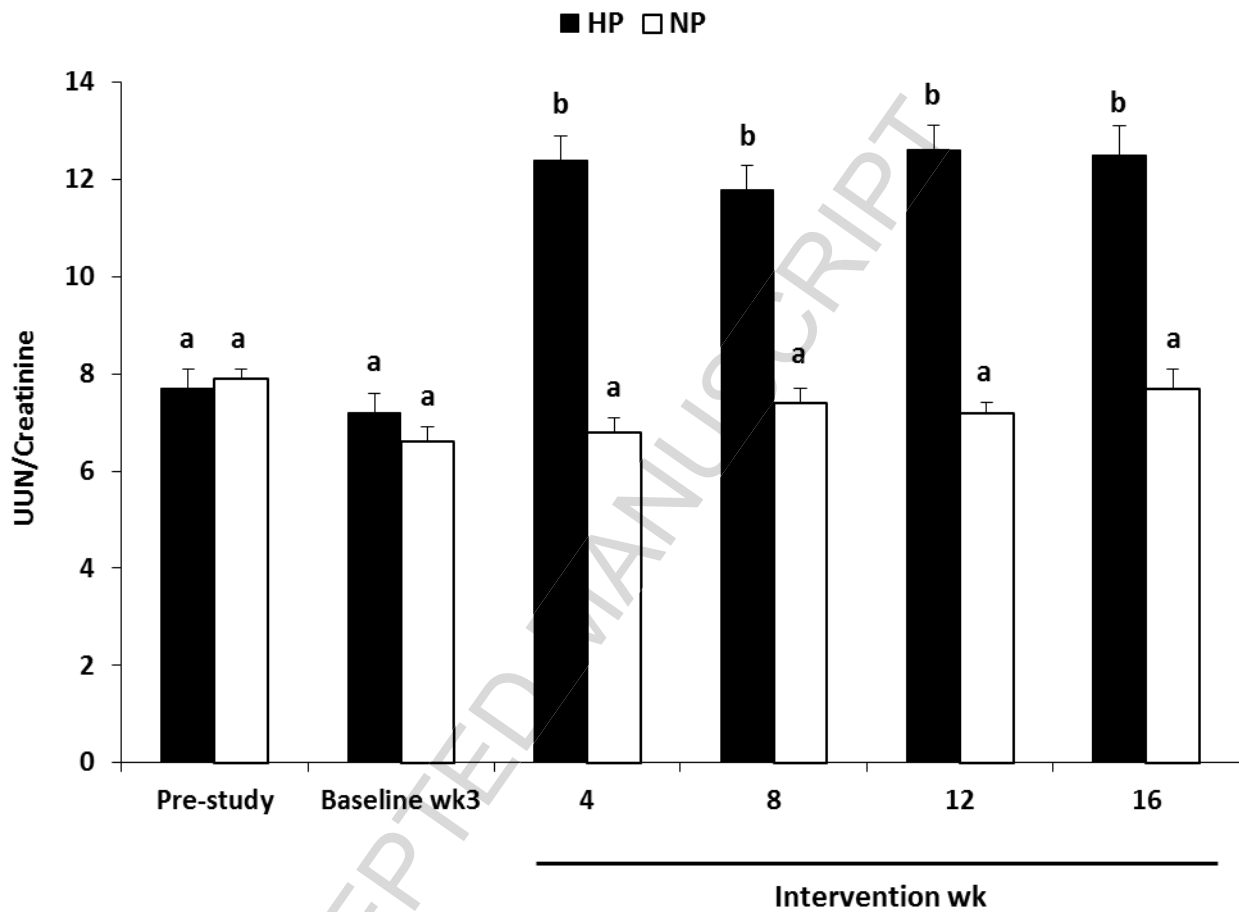
Results are reported as means±SE. Values without a common letter are significantly different at each intervention period, $p < 0.05$. HP, high protein; NP, normal protein; UUN, urinary urea nitrogen.

Figure 5. Changes in plasma lipids and lipoproteins
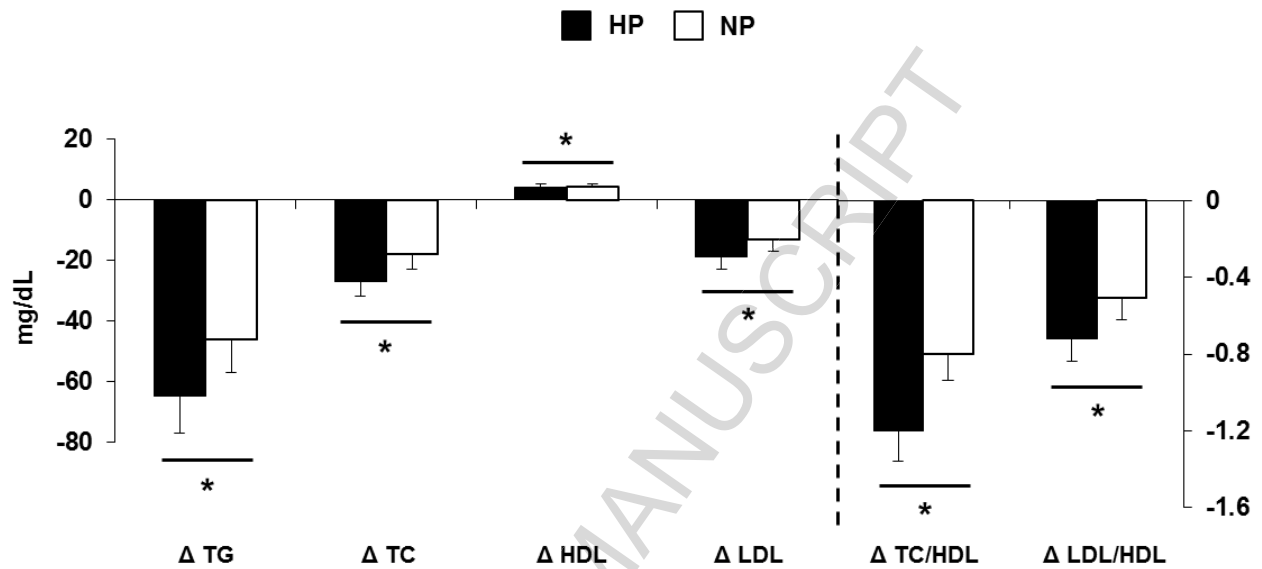
Results are reported as lsmeans±SE. *A main effect of time (p<0.05). Analyses are adjusted for age and gender. HP, high protein; NP, normal protein; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol.

Figure 6. Changes in monocyte subpopulations

Results are reported as lsmeans±SE. *A main effect of time (p<0.05). Analyses are adjusted for age and gender. HP, high protein; NP, normal protein.