SHORT-TERM HIGH-FAT DIET INCREASES MACROPHAGE MARKERS IN SKELETAL MUSCLE ACCOMPANIED BY IMPAIRED INSULIN SIGNALING IN HEALTHY MALE SUBJECTS

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ABBREVATIONS

CETP	cholesteryl ester transfer protein
hsCRP	high sensitive C-reactive protein
(F)FA	(free) fatty acid
GLUT-4	glucose transporter 4
GYS1	glycogen synthase 1
HFD	high-fat diet
HFHC	high-fat high calorie
INSR	insulin receptor
IR	insulin resistance
RT-MLPA	dual color reverse transcriptase multiplex ligation-dependent probe amplification
SLC2A4	solute carrier family 2
TBC1D1	TBC1 domain family member 1
TBC1D4	TBC1 domain family member 4
TG	triglyceride
(s)WAT	(subcutaneous) white adipose tissue

ABSTRACT

Recent studies have demonstrated elevated macrophage markers in skeletal muscle of obese subjects, that inversely related to insulin sensitivity. The aim of the present study was to investigate whether short-term high-fat high calorie (HFHC) diet already increases macrophage markers and affects glucose metabolism in skeletal muscle of healthy lean subjects. To this end, 24 healthy lean young male subjects received a 5-day HFHC diet. Before and after the diet, muscle and subcutaneous white adipose tissue (sWAT) biopsies were taken and mRNA expression levels of relevant genes, plasma glucose, insulin, C-peptide and cholesteryl ester transfer protein (CETP) levels were measured. 5 days of HFHC diet markedly increased mRNA expression of the general macrophage markers *CD68* (3.7-fold, $P<0.01$) and *CD14* (3.2-fold, $P<0.01$), as well as the M1 macrophage marker *MARCO* (11.2-fold, $P<0.05$) in muscle. This was accompanied by downregulation of *TBC1D1, SLC2A4* and *GYS1* mRNA expression, all involved in uptake and storage of glucose, and elevation of plasma glucose (+4%, $P<0.001$) and insulin (+55%, $P<0.001$) levels together with HOMA-IR (+48%, $P<0.001$), suggesting development of insulin resistance. Furthermore, plasma CETP levels, a marker of liver macrophage content, were increased (+21%, $P<0.001$) while macrophage content in sWAT remained unchanged. Short-term HFHC diet increases expression of macrophage markers in skeletal muscle, but not sWAT, of healthy male subjects accompanied by reduced markers of insulin signalling and development of insulin resistance. Therefore, recruitment of macrophages into muscle may be an early event in development of insulin resistance in the course of obesity.

INTRODUCTION

Skeletal muscle is the primary site of dietary glucose disposal *in vivo*. Insulin stimulates glucose uptake into muscle through an elaborate signaling cascade eventually resulting in mobilization of glucose transporter 4 (GLUT-4) channels to the plasma membrane that mediate glucose uptake (1). High-fat diet (HFD) feeding impairs whole-body insulin sensitivity and substrate homeostasis in both mice and humans (2,3). Skeletal muscle insulin resistance (IR) is the major contributor to development of whole-body IR (4) but the exact underlying molecular mechanism remains to be elucidated, although various hypotheses have been proposed.

First, lipid accumulation inside muscle cells and the formation of subsequent lipid metabolites *(e.g.* diacylglycerols, ceramides and acylcarnitines) contribute to muscle IR by interfering with the insulin signaling cascade (5).

Second, fatty acids (FAs) trigger activation of inflammatory signals in myocytes, and also in innate immune cells such as macrophages, leading to the release of pro-inflammatory cytokines that result in low-grade systemic inflammation (6,7). *In vitro* studies support this concept, showing that FAs promote release of pro-inflammatory cytokines by macrophages that, in turn, induce IR in skeletal muscle cells (8). Of note, cytokines can directly influence insulin actions on muscle. For example, the pro-inflammatory cytokine TNFα caused IR and impaired glucose uptake in primary human myocytes (9).

A third mechanism underlying the development of muscle IR in response to HFD may involve recruitment of macrophages into the muscle tissue itself. Hong et al (10) showed that HFD increased the number of M1-activated (CD11c+) macrophages in skeletal muscle in mice. Of note, this was corrected by transgenic overexpression of the anti-inflammatory cytokine IL-10 in skeletal muscle, which also improved whole-body insulin sensitivity. In humans, Varma et al (8) showed that the skeletal muscle from obese subjects contained 2.5-fold higher CD68+ macrophage numbers in skeletal muscle compared to lean subjects. Strikingly, their study showed that macrophage content in muscle was strongly associated with BMI and inversely related to insulin sensitivity.

Taken together, these data suggest that HFD results in recruitment of pro-inflammatory macrophages in muscle which contribute to the development of muscle IR by releasing pro-inflammatory cytokines. However, the timing at which macrophages are recruited into skeletal muscle in the time course of HFD-induced obesity and the possible involvement of other, adaptive, immune cell types is currently unknown and may either be an early event or a late consequence when chronic low-grade inflammation has already developed. To investigate this issue, we subjected healthy, male lean subjects for 5 days to a high-fat high calorie (HFHC) diet and studied expression of markers for macrophages, T cells, pattern recognition receptors, as well as markers for glucose metabolism, in skeletal muscle biopsies before and after the diet. Furthermore, to investigate whether short-term HFD results in influx of macrophages in other tissues besides muscle, we investigated expression of macrophage markers in subcutaneous white adipose tissue (sWAT) biopsies as well as plasma cholesteryl ester transfer protein (CETP) levels as a marker of the liver macrophage content (11).

METHODS AND MATERIALS

Study participants

24 Dutch, lean (BMI < 25 kg/m^2) and healthy males of Caucasian (n=12) and South Asian (n=12) ethnicity between 19 and 25 years of age and with a positive family history of type 2 diabetes were enrolled via local advertisements. All subjects underwent a medical screening including their medical history, a physical examination, blood chemistry tests and an oral glucose tolerance test to exclude individuals with type 2 diabetes according to the American Diabetes Association (ADA) 2010 criteria. Other exclusion criteria were rigorous exercise, smoking and recent body weight change. The present study was approved by the Medical Ethical Committee of the Leiden University Medical Centre and performed in accordance with the principles of the revised Declaration of Helsinki. All volunteers gave written informed consent before participation.

Study design

Subjects were studied before and after a 5-day HFHC diet, consisting of the subject's regular diet, supplemented with 375 ml of cream per day (=1275 kcal/day extra containing 94% fat). They were instructed not to alter life style habits, and not to perform physical activity in the last 48 hours prior to the study days. Furthermore, subjects were asked to keep a food diary before and during the HFHC diet to estimate normal dietary intake, and to check for compliance and compensation behavior respectively. Diaries were entered and analyzed using a specialized internet application (http://www.dieetinzicht.nl, Dutch). Magnetic resonance (MR) studies were performed shortly before and on the fifth day of the HFHC diet. In addition, one day before and one day after the diet anthropometric measurements were performed and muscle and subcutaneous white adipose tissue biopsies were obtained from all subjects in fasted condition.

MR studies

Hepatic triglyceride content was assessed by proton MR-spectroscopy (^{1}H-MRS; Gyroscan ACS-NT15; Philips, The Netherlands) in postprandial state (five hours after the last meal), as previously described (12). Vertebra Th12 was used as a marker to ascertain the same position of the 8-ml-voxel at both study days. A spectrum with water suppression as internal standard was obtained, and 64 averages were collected without water suppression. The spectra were fitted using Java-base MR user interface software (jMRUI version 2.2) (12). The percentage of hepatic triglyceride (TG) signals was calculated as: (signal amplitude hepatic TG/ signal amplitude water) x 100.

Anthropometric measurements

Anthropometric measurements were performed after an overnight fast. Body composition (fat and lean body mass) was assessed by bioelectrical impedance analysis (BIA; Bodystat® 1500, Bodystat Ltd., Douglas, UK).

Skeletal muscle biopsies
Muscle biopsies from the *m. vastus lateralis* (approx. 75-100 mg) were collected one day before and one day after the diet intervention in fasted condition under localized anesthesia, using a modified Bergström needle, as previously described (13). Muscle samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

White adipose tissue biopsies
Subcutaneous fat biopsies were obtained from the umbilical region in fasted condition under localized anesthesia, using a syringe with needle while applying suction. Fat samples were immediately submersed into a medium (DMEM/F12 + Glutamax) until further analysis. Of note, analysis was done in a subset of subjects (n=8) due to limited availability of tissue in lean subjects.

Laboratory analysis
Fasting serum glucose, total cholesterol, HDL-cholesterol and triglycerides (TG) were measured on a Modular P800 analyzer (Roche, Almere, The Netherlands). LDL-cholesterol was calculated according to Friedewald's formula (14). Serum insulin and C-peptide levels were analyzed on an Immulite 2500 (Siemens, The Netherlands). The HOMA-IR index was calculated using the formula: fasting insulin (pmol/L) x fasting glucose (mmol/L)/22.5. Plasma free fatty acid (FFA) concentrations were determined by a colorimetric method (Wako Chemicals, Germany). ELISA kits were used to measure serum levels of high-sensitive C-reactive protein (hsCRP) (Meso Scale Discovery, Gaithersburg, USA), adiponectin (Cayman Chemical, Ann Arbor, USA) and CETP concentration (ALPCO Diagnostics, Salem, USA) according to the manufacturers' instructions.

RNA isolation
Total RNA was isolated from skeletal muscle biopsies (approx. 25-30 mg) using the phenol-chloroform extraction method (Tripure RNA Isolation reagent, Roche, Germany) and treated with a DNAse kit (TURBO DNAse, Life Technologies, The Netherlands) according to the manufacturer's instruction. Amount of RNA was determined by NanoDrop.

cDNA synthesis and real-time PCR
For RT-PCR, first-strand cDNA was synthesized from 1 µg total RNA using a Superscript first strand synthesis kit (Invitrogen, The Netherlands). Real-time PCR was carried out on the IQ5 PCR machine (Bio-Rad) using the Sensimix SYBRGreen RT-PCR mix (Quantace). Melt curve analysis was included to assure a single PCR product was formed. Expression levels were normalized to ribosomal protein S18 (RPS18). Primer sequences are listed in **SUPPLEMENTARY TABLE 1.**

dcRT-MLPA assay
A dual-color reverse transcriptase multiplex ligation-dependent probe amplification (dcRT-MLPA) assay was performed as described previously (15). Briefly, for each target-specific

sequence, a specific RT primer was designed located immediately downstream of the left and right hand half-probe target sequence. Following reverse transcription, left and right hand half-probes were hybridized to the cDNA at 60°C overnight. Annealed half-probes were ligated and subsequently amplified by PCR (33 cycles of 30 s at 95°C, 30 s at 58°C and 60 s at 72°C, followed by 1 cycle of 20 min at 72°C). PCR amplification products were 1:10 diluted in HiDi formamide-containing 400HD ROX size standard and analyzed on an Applied Biosystems 3730 capillary sequencer in GeneScan mode (Applied Biosystems).

Trace data were analyzed using the GeneMapper software package (Applied Biosystems). The areas of each assigned peak (in arbitrary units) were exported for further analysis in Microsoft Excel spreadsheet software. Data were normalized to beta-2-microglobulin (B2M) and signals below the threshold value for noise cutoff in GeneMapper (log2 transformed peak area 7.64) were assigned the threshold value for noise cutoff

Immunohistochemical stainings

Formalin-fixed-paraffin-embedded subcutaneous adipose tissue sections were used for immunohistochemistry of CD68. Antigens were retrieved using citrate buffer. The primary antibody was mouse-anti-human CD68 (1:800 dilution, clone KP1, from Dako, Glostrup, Denmark). Staining and counterstaining was done with Nova Red (Vector labs, Brunschwig Chemie, Amsterdam, The Netherlands) and haematoxylin, respectively. Crown-like structures (CLS) were counted according to the criterion that a CLS consisted of three or more CD68 positive cells surrounding an adipocyte.

Statistical analysis

The data of South Asian and Caucasian subjects were pooled for all analyses since no significant differences were observed between ethnicities for baseline values or diet effects. Data are presented as mean ± SEM. Paired T-tests were applied to assess mean differences before and after the diet intervention. Significance level was set at $P<0.05$. Statistical analyses were performed using SPSS for Windows version 20.0 (IBM, USA).

RESULTS

Clinical characteristics

Clinical characteristics are shown in **TABLE 1**. Mean age was 22.1±0.4 years and mean BMI was 21.5±0.4 kg/cm^2. Five days of HFHC diet resulted in a small but significant increase in body weight (+0.7%, $P<0.01$) and BMI (+0.9%, $P<0.01$), while waist circumference and percentage of fat mass remained unchanged. Although plasma FFA, serum TG and total cholesterol levels did not change significantly upon the HFHC diet, HDL-C and LDL-C did increase (both +9%, $P<0.01$). Furthermore, the HFHC diet significantly increased fasting glucose (+4%), insulin (+55%) and C-peptide (+29%) levels, as well as the HOMA-IR index (+ 48%) (all $P<0.001$).

HFHC diet and markers of glucose uptake/metabolism and muscle inflammation

The HFHC diet did not affect mRNA expression of the insulin receptor *(INSR)* and its downstream signaling target *TBC1D4* (encoding AS160) in muscle biopsies **(FIG 1A)**. However, a diminished expression of TBC1D1 (-23%, P<0.05), *SLC2A4* encoding GLUT-4 (-34%, P<0.001) and GYS-1 (-35%, P<0.001), all involved in uptake and storage of glucose, was evident.

Using the dcRT-MLPA assay, we measured mRNA expression of a large panel of inflammatory genes, including markers for innate and adaptive immune cells, pattern recognition receptors and cytokines in muscle biopsies before and after the HFHC diet **(TABLE 2)**. Of note, the HFHC diet significantly upregulated the expression of *CD14* (+118%, P<0.05), a general macrophage marker, and the expression of *MARCO* (+415%, P<0.05), a scavenger receptor that is mainly present on pro-inflammatory M1 macrophages (16). In line with this, expression of the anti-inflammatory cytokine *IL10* tended to be downregulated (-27%, P=0.10).

Furthermore, expression of the general T-cell marker *CD3* was undetectable in the muscle biopsies, pointing to absence or very low presence of T-cells. Expression of *CD4,* a T-helper cell marker that is also expressed by innate immune cells such as monocytes and macro-

TABLE 1 - **Anthropometrics and metabolic characteristics before and after a 5-day HFHC diet in healthy, young male subjects**

	Before HFHC diet (n= 24)	After HFHC diet (n=24)
Age (years)	22.1 ± 0.4	
Length (m)	1.79 ± 0.01	
Weight (kg)	69.1 ± 1.9	**69.6 ± 1.9****
BMI (kg/cm2)	21.5 ± 0.4	**21.7 ± 0.4****
Waist (cm)	80.1 ± 1.5	80.8 ± 1.7
Fat mass (%)	13.2 ± 0.7	13.0 ± 0.7
Total cholesterol (mmol/L)	4.16 ± 0.19	4.51 ± 0.17
HDL-C (mmol/L)	1.22 ± 0.05	**1.33 ± 0.04****
LDL-C (mmol/L)	2.51 ± 0.17	**2.74 ± 0.15****
Triglycerides (mmol/L)	0.96 ± 0.08	0.97 ± 0.08
FFA (mmol/L)	0.49 ± 0.03	0.48 ± 0.03
Glucose (mmol/L)	5.17 ± 0.06	**5.37 ± 0.06*****
Insulin (pmol/L)	4.53 ± 1.00	**7.02 ± 1.16*****
C-peptide (nmol/L)	0.49 ± 0.03	**0.63 ± 0.04*****
HOMA-IR	1.62 ± 0.26	**2.39 ± 0.32*****
hsCRP (µg/mL)	0.94 ± 0.23	1.16 ± 0.24
Adiponectin (µg/mL)	6.27 ± 0.63	6.58 ± 0.50
Hepatic TG content (%)	1.57 ± 0.27	**3.43 ± 0.49*****
CETP mass (µg/mL)	1.69 ± 0.09	**2.02 ± 0.10*****

Data are presented as mean ± SEM. ** P<0.01, *** P<0.001 vs. before diet. HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; FFA, free fatty acid; hsCRP, high-sensitive C-reactive protein; TG, triglyceride; CETP, cholesteryl ester transfer protein.

TABLE 2 - Expression of adaptive and innate immune markers in muscle biopsies before and after a 5-day HFHC diet in healthy male subjects

Gene	Baseline	HFHC (fold change vs baseline)	P-value
Immune cell subset markers			
CD14	**1.00 ± 0.16**	**2.18 ± 0.23**	**0.04***
CD163	1.00 ± 0.10	0.84 ± 0.15	0.31
NCAM1	1.00 ± 0.14	1.15 ± 0.13	0.37
CD19	1.00 ± 0.12	1.09 ± 0.16	0.61
BLR1	1.00 ± 0.15	0.84 ± 0.14	0.42
FCGR1A#	1.00 ± 0.00	1.68 ± 0.26	0.13
T cell subsets			
CD3E#	1.00 ± 0.00	1.01 ± 0.01	0.33
CD4	**1.00 ± 0.09**	**1.48 ± 0.15**	**0.04***
CD8A	1.00 ± 0.14	0.89 ± 0.15	0.61
IL7-R	1.00 ± 0.06	0.93 ± 0.10	0.53
CCR7	1.00 ± 0.08	0.87 ± 0.11	0.26
Th1 response			
CXCL10	1.00 ± 0.10	0.98 ± 0.11	0.88
IFNG	1.00 ± 0.27	1.62 ± 0.28	0.28
IL-2	1.00 ± 0.17	1.06 ± 0.17	0.84
IL-1B	1.00 ± 0.13	0.93 ± 0.15	0.78
TNF	1.00 ± 0.08	1.09 ± 0.10	0.52
Th2 response			
IL-4#	1.00 ± 0.29	0.79 ± 0.24	0.56
IL-4d2	1.00 ± 0.35	0.79 ± 0.35	0.65
IL-5	1.00 ± 0.24	1.04 ± 0.24	1.00
IL-6	1.00 ± 0.11	1.08 ± 0.14	0.70
IL-9#	1.00 ± 0.00	1.17 ± 0.10	0.16
IL-10	1.00 ± 0.17	0.73 ± 0.19	0.13
IL-13	1.00 ± 0.45	1.94 ± 0.28	0.26
Treg markers			
CCL4	1.00 ± 0.16	1.11 ± 0.15	0.80
CTLA4	1.00 ± 0.13	0.88 ± 0.13	0.38
LAG3	1.00 ± 0.29	0.43 ± 0.42	0.08
TGF-B1	1.00 ± 0.13	1.12 ± 0.11	0.42
Key MF1 and MF2 cytokines			
IL-12A	1.00 ± 0.32	0.89 ± 0.46	0.82
IL-12B	1.00 ± 0.39	1.10 ± 0.36	0.85
IL-23A	1.00 ± 0.08	0.81 ± 0.13	0.22
CCL2#	1.00 ± 0.44	1.78 ± 0.40	0.37
CCL5	1.00 ± 0.09	0.90 ± 0.10	0.51

Gene	Baseline	HFHC (fold change vs baseline)	P-value
CCL19	1.00 ± 0.11	1.07 ± 0.13	0.66
CCL22	1.00 ± 0.08	0.85 ± 0.06	0.16
CXCL13#	1.00 ± 0.40	0.97 ± 0.38	0.96
Scavenger receptors			
MARCO	**1.00 ± 0.23**	**5.15 ± 0.37**	**0.04***
Pattern recognition receptors			
TLR1#	1.00 ± 0.00	1.55 ± 0.36	0.33
TLR2#	1.00 ± 0.51	0.17 ± 0.00	0.11
TLR3	1.00 ± 0.11	1.07 ± 0.14	0.68
TLR4	1.00 ± 0.29	1.20 ± 0.33	0.67
TLR5	1.00 ± 0.57	0.46 ± 0.65	0.33
TLR6	1.00 ± 0.37	1.28 ± 0.32	0.64
TLR7	1.00 ± 0.12	0.96 ± 0.15	0.83
TLR8	1.00 ± 0.10	0.96 ± 0.17	0.85
TLR9	1.00 ± 0.11	0.79 ± 0.14	0.21
TLR10##	1.00 ± 0.00	2.36 ± 0.59	0.33
NOD1	1.00 ± 0.24	1.06 ± 0.15	0.83
NOD2#	1.00 ± 0.00	1.33 ± 0.26	0.33
MRC1	1.00 ± 0.23	1.63 ± 0.18	0.06
MRC2	1.00 ± 0.05	0.91 ± 0.11	0.48
CD209	1.00 ± 0.14	1.02 ± 0.16	0.94
CLEC7A	1.00 ± 0.17	1.23 ± 0.19	0.38
Inflammasome components			
NLRP1#	1.00 ± 0.51	0.21 ± 0.00	0.13
NLRP2	1.00 ± 0.10	1.00 ± 0.15	0.99
NLRP3#	1.00 ± 0.42	0.85 ± 0.32	0.77
NLRP4	1.00 ± 0.16	0.86 ± 0.22	0.56
NLRP7	1.00 ± 0.37	0.88 ± 0.35	0.78
NLRC4	1.00 ± 0.05	0.89 ± 0.10	0.37

Data are presented as mean ± SEM and expressed relative to baseline. *P<0.05 vs. before diet. # Low expression of gene.

phages (17), was detectable and increased upon the HFHC diet (+48%, P<0.05). Given the absence of CD3 in the muscle biopsies, the increase in CD4 expression probably reflects increased expression by innate immune cells. Other inflammatory markers remained unaffected.

Thus, these data suggested that upon a short-term HFHC diet, especially markers of innate immunity were upregulated in muscle. We confirmed these findings by performing RT-PCR analyses on several macrophage markers in the muscle biopsies **(FIG 1B)**. More specifically, the HFHC diet upregulated the general macrophage markers *CD68* (3.7-fold, P<0.01) and *CD14* (3.2-fold, P<0.01), as well as the M1 marker *MARCO* (11.2-fold, P<0.05). Hence, short-term HFHC diet resulted in increased expression of pro-inflammatory M1 macrophages in muscle.

HFHC diet and markers of inflammation in other tissues

Since HFD-induced obesity is often associated with influx of pro-inflammatory macrophages and appearance of crown-like structures (CLS) in WAT (18), we next investigated whether markers of macrophage infiltration were also evident in WAT after 5 days of HFHC diet in healthy lean subjects. Due to the very small amount of tissue, we could only perform immunohistological staining of CD68 in subcutaneous WAT taken before and after the diet intervention in a subset of subjects. At baseline, solitary macrophages were identified sporadically in the WAT biopsies, while no CLS were observed. After the HFHC diet, influx of solitary macrophages increased in only a few subjects as did the appearance of CLS in WAT **(SUPPLEMENTARY FIGURE 1A-B)**, however, these effects were not statistically significant. Further-

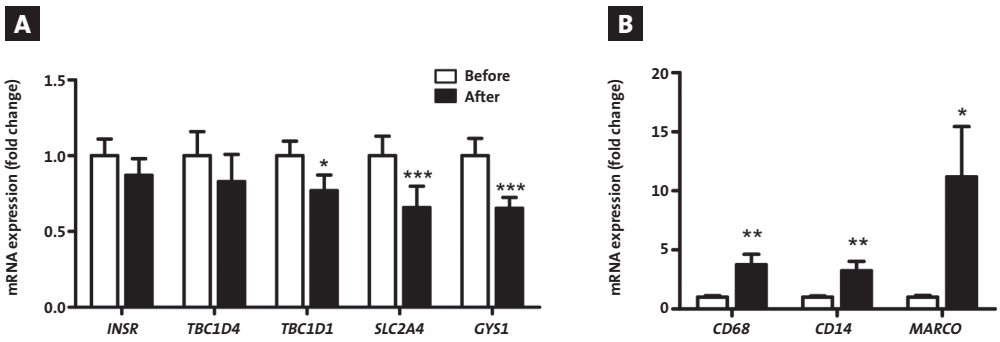

FIGURE 1 - Effect of short-term HFHC diet on expression of insulin signaling and macrophage markers in muscle in healthy male subjects. mRNA expression levels of genes related to glucose uptake/metabolism **A** and macrophage markers **B** were measured in skeletal muscle biopsies of healthy male subjects (n=24) obtained before (white bars) and after (black bars) a 5-day HFHC diet. Expression levels are normalized to the housekeeping gene *S18* and expressed as fold change compared to baseline as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. before diet.

HFHC, high-fat high calorie. INSR, insulin receptor. TBC1D4, TBC1 domain family member 4. TBC1D1, TBC1 domain family member 1. SLC2A4 solute carrier family 2. GYS1, glycogen synthase 1.

more, plasma adiponectin, an adipocyte-derived hormone that is suggested to play a role in suppression of the development of obesity and IR (19), did not change upon the dietary intervention **(TABLE 1)**.

The HFHC diet increased the liver triglyceride (TG) content (+118%, P<0.001; **TABLE 1**). For obvious reasons we could not obtain liver biopsies from our healthy subjects to measure macrophage content. However, we have recently shown that plasma CETP levels highly correlate with levels of general macrophage markers in liver biopsies (Wang and Rensen et al, *unpublished)* and therefore, we measured plasma CETP levels as a marker of liver macrophage content. Intriguingly, plasma CETP levels increased upon the HFHC diet (+21%, P<0.001; **TABLE 1)**, suggesting influx of macrophages into the liver. Furthermore, after the HFHC diet plasma CRP levels tended to increase (+24%, P=0.06; **TABLE 1)**, pointing to low-grade systemic inflammation.

DISCUSSION

Recent studies have detected elevated numbers of macrophage markers in skeletal muscle during chronic HFD feeding and obesity, but the timing at which these macrophages are recruited and their role in the development of muscle and whole-body IR is unknown. The present study demonstrates that only 5 days of HFHC diet resulted in a marked increase in expression of macrophage markers in muscle of healthy lean male subjects. Moreover, this was accompanied by downregulation of genes involved in glucose metabolism and elevation of fasting plasma glucose and insulin levels and HOMA-IR index. Of note, plasma CETP levels, which were previously shown by our group to correlate with liver macrophage content, increased while macrophage content in the subcutaneous WAT depot remained unchanged.

As in other organs, resident macrophages are present in human skeletal muscle (8) where they contribute to regeneration and revascularization in case of damage (20). In accordance with these data, we found that the general macrophage markers CD14 and CD68 were significantly expressed in skeletal muscle of healthy lean men. Of note, the homeostatic functions of muscle macrophages are exerted only when the macrophages are in their M2 (anti-inflammatory) phenotypic polarization stage (21,22). In response to different stimuli, such as circulating FA, macrophages are polarized towards an M1-like (inflammatory) phenotype, leading to release of pro-inflammatory cytokines which can induce IR in myocytes (8). Indeed, previous mouse and human studies have repeatedly observed increased numbers of activated M1 (CD11c+) macrophages in skeletal muscle tissue in the context of obesity and IR (23-26). Intriguingly, our data demonstrate that the increased expression of M1 macrophages in skeletal muscle occurs already after short-term HFD and even in healthy young subjects, supporting the concept that recruitment of M1 macrophages into skeletal muscle is an early event in the time course of HFD-induced obesity.

What would be the trigger of the extensive increase in macrophage markers in muscle

and does this mainly reflect activation of resident macrophages or increased influx from blood-derived monocytes? We speculate that both mechanisms are involved. The fact that the expression of general macrophage markers (CD68 and CD14) was increased, is most easily explained by an increased influx of these cells into muscle, rather than by upregulation of the expression of these genes in resident macrophages. The trigger that stimulated this influx is likely the high load of saturated FAs ingested with the HFHC diet. Saturated FAs can signal via Toll-like receptors (TLRs), particularly TLR-4 that is present on both macrophages and myocytes (27). Activation of TLR-4 stimulates the transcription and release of various chemokines and pro-inflammatory cytokines, such as MCP-1 or tumor necrosis factor-α (TNF-α), which attract other macrophages and may also directly impair insulin signaling in skeletal muscle (5; 28,29). In the present study, we did not find effects of the HFHC diet on gene expression of Toll-like receptors or pro-inflammatory cytokines such as TNF-α in skeletal muscle. However, we cannot exclude that either local release of pro-inflammatory cytokines or activation of TLR-4-driven signaling pathways might have occurred in this tissue.

Intriguingly, while we found that expression of macrophage markers was markedly increased in skeletal muscle upon the HFHC diet, no significant increase could be identified in the subcutaneous WAT depot. This might be in part due to the fact that visceral adipose tissue (VAT) seems to be more closely associated with the inflammatory state than subcutaneous adipose tissue (30). Furthermore, muscle cells might be more prone to release macrophage attractant factors in the presence of pro-inflammatory stimuli compared to adipocytes. Indeed, a recent study demonstrated that the presence of both macrophages and the saturated FA palmitate exerted a synergistic effect on MCP-1 release by muscle cells, resulting in greater attraction of macrophages (8). To our knowledge, such a positive feedback-loop has not been demonstrated for adipocytes.

Besides a large increase in macrophage markers in muscle upon the HFHC diet, the increased plasma CETP levels suggests increased hepatic macrophage content as well. Our group has recently shown that the hepatic macrophage is the main producer of plasma CETP (Wang et al., *unpublished),* meaning that an increase in plasma CETP levels reflects an increase in hepatic macrophages. Interestingly, various studies have shown that pro-inflammatory cytokines also impaired insulin signaling in hepatocytes (31). Thus, it is tempting to speculate that dietary saturated FAs may have resulted in influx of pro-inflammatory M1 macrophages in the liver, leading to release of pro-inflammatory cytokines and subsequent impairment of hepatic insulin signaling. This mechanism may also explain the previously found reduced suppression of endogenous glucose production in response to 5 days of HFHC diet (32).

A potential limitation of the current study could be that we determined immune cell markers via MLPA-assay and RT-PCR instead of performing fluorescence-activated cell sorting (FACS) analyses, a method by which true cell counts are determined. However, performing FACS analyses would require relatively large amounts of muscle tissues, the collection of which was not feasible in the current study.

In conclusion, we show that 5 days of HFHC diet resulted in a marked increase in gene expression of M1 macrophage markers in skeletal muscle of healthy lean male subjects, a

feature associated with apparent impairment in whole-body insulin sensitivity and glucose homeostasis. Future studies should be directed at unraveling the precise contribution of muscle macrophages in the development of peripheral IR, with the ultimate goal to develop novel therapeutic targets that decrease inflammation-induced IR without interfering with all innate immune functions.

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The authors have no potential conflicts of interest relevant to this article.

MRB, LEHB, AEM, IMJ and PCNR designed research; MRB, LEHB, MCH, EQ, LvB, YW, VvH and BG conducted research; MRB, LEHB, and MCH analyzed data; MRB, LEHB, BG, IMJ, and PCNR wrote the paper; MCH, EQ, LvB, YW, VvH, AEM, THMO, KWvD, BG, IMJ and PCNR contributed to the discussion, and reviewed and edited the manuscript; MRB and LEHB had primary responsibility for final content. All authors read and approved the final manuscript.

L.E.H.B, M.R.B, I.M.J and P.C.N.R. are the guarantors of this work and, as such, have full access to all the data generated in the framework of the study and take responsibility for their integrity and the accuracy of their analysis.

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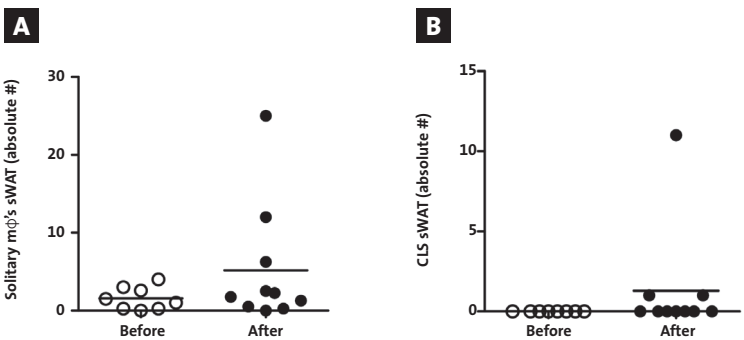
SUPPLEMENTARY DATA

SUPPLEMENTARY TABLE 1 - **Primer sequences**

Gene	Forward primer	Reverse primer
CD14	CGCTCCGAGATGCATGTG	AGCCCAGCGAACGACAGA
CD68	AGCCCAGATTCAGATTCGAG	GGGAATGAGAGAAGCAGGTG
GYS1	TGGGGCTACACACCGGCTGA	GCGGAACCGCCGGTCAAGAA
INSR	GGGCAACGGCTCTTGGACGG	CGGCCCATCTGGCTGCCTCTT
MARCO	CTGGAGAACACCTGGCTCA	CCCTTGTGACCTTGAAGAC
RPS18	GGACCTGGCTGTATTTTCCA	GAGGATGAGGTGGAACGTGT
SLC2A4 (GLUT-4)	GCTACCTCTACATCATCCAGAATCTC	CCAGAAACATCGGCCCA
TBC1D1	GGGCAAGGTGTGCCACGTCA	TCGCATGCTGCTGGGAAGTCA
TBC1D4 (AS160)	GGCTGGAGTCCTGCTTCTGCAC	GCTGGTACATTTGAATCTGCAGCGA



SUPPLEMENTARY FIGURE 1 - Effect of short-term HFHC diet on macrophage content in subcutaneous white adipose tissue in healthy male subjects. The number of solitary macrophages and CLS were counted following a CD68 (macrophage) staining in sWAT biopsies of healthy male subjects (n=8) obtained before (open dots) and after (closed dots) a 5-day HFHC diet. Counts represent absolute numbers.

HFHC, high-fat high calorie. CLS, crown-like structure. sWAT, subcutaneous white adipose tissue.

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