Food & Function

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Journal:	*Food & Function*
Manuscript ID	FO-ART-03-2016-000381
Article Type:	Paper
Date Submitted by the Author:	17-Mar-2016
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Niacin and olive oil promote the skewing to M2 phenotype in bone marrow-derived macrophages of mice with metabolic syndrome

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Short title: Niacin and olive oil promote M2 in BMDM

ABSTRACT

Metabolic syndrome (MetS) is associated with obesity, dyslipemia, type 2 diabetes and chronic low-grade inflammation. The aim of this study was to determine the role of high-fat low-cholesterol diets (HFLCDs) rich in SFAs (HFLCD-SFAs), MUFAs (HFLCD-MUFAs) or MUFAs plus omega-3 long-chain PUFAs (HFLCD-PUFAs) on polarisation and inflammatory potential in bone marrow-derived macrophages (BMDMs) from niacin (NA)-treated $Lep^{ob/ob}LDLR^{-/-}$ mice. Animals fed with HFLCD-SFAs had increased weight and serum triglycerides, and their BMDMs accumulated triglycerides over the animals fed with HFLCD-MUFAs or -PUFAs. Furthermore, BMDMs from animals fed with HFLCD-SFAs were polarised towards M1 phenotype with functional competence to produce pro-inflammatory cytokines, whereas BMDMs from animals fed with HFLCD-MUFAs or -PUFAs were skewed to anti-inflammatory M2 phenotype. These findings open opportunities for developing novel nutritional strategies with olive oil as the most important dietary source of MUFAs (notably oleic acid) to prevent development and progression of metabolic complications in the NA-treated MetS.

Keywords: Olive oil, niacin, bone marrow-derived macrophages, metabolic syndrome, inflammation

INTRODUCTION

Niacin (NA, also commonly known as nicotinic acid or vitamin B3) is a water-soluble vitamin that is converted in vivo to nicotinamide adenine dinucleotide (NAD^+), a coenzyme involved in the lipid catabolism.[1] NA has been established as a broad-spectrum lipid-modulating drug.[2] However, it is becoming increasingly evident that NA possesses pleiotropic effects upon attenuating obesity-induced inflammation.[3,4] During weight gain, white adipose tissue (WAT) resident macrophages may undergo a 'phenotypic switch' from an anti-inflammatory M2 to a pro-inflammatory M1 phenotype, linking to the emergence of insulin resistance and metabolic syndrome (MetS)-related disorders.[5] Nutritional-based strategy to counteract these complications is to decrease the consumption of energy-dense high-fat diets.[6] However, it is unknown whether the type of dietary fats plays any role on macrophage plasticity during obesity-related inflammation in association with NA treatment in MetS.

MATERIALS AND METHODS

Fatty acid composition of dietary fats

The fatty acid (FA) composition of dietary fats [cow's milk cream, rich in saturated FAs (SFAs); refined olive oil, rich in monounsaturated FAs (MUFAs); and refined olive oil plus eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), rich in MUFAs and omega-3 long-chain polyunsaturated FAs (PUFAs)] was determined by the method described in EEC/796/2002,[7] using a gas chromatography system (HP-5890, Hewlett-Packard) equipped with flame ionization detector and a SP-2380 capillary column (Supelco, 30 m x 0.32 mm) packed with cyan propyl siloxane (0.25

μm). The initial column temperature was 165 ºC, which was held for 10 min, then programmed from 165 ºC to 200 ºC at 1.5 ºC/min. Injector and detector temperature were 250 ºC, with the carrier gas H_2. The FA composition of different dietary fats is detailed in **Table 1**.

Animal diets and experimental design

Male $Lep^{ob/ob}LDLR^{-/-}$ mice bread onto a C57BL/6J background (B6.Cg-Lepob Ldlrtm1Her/J, The Jackson Laboratory, Bar Harbor, ME) was used for the study. These mice are obese and develop plasma lipid alterations that closely reflect MetS-related hyperlipidemia.[8] All diets were prepared by Panlab Laboratoires (SAFE, Augy, France) and presented as pellets to the animals. Mice received one of the following diets for 8 weeks: a standard normal-fat diet (low-fat low-cholesterol diet, LFLCD) containing 3% energy as fat, used as control, or high-fat low-cholesterol diets (HFLCDs), which contained 24% energy as fat. All the diets were based on the standard rodent diet A04-10, containing 0.01% cholesterol, 20 mg/kg BHT, and 3% binder. Three different HFLCDs were prepared by replacing the fat source from A04-10 diet by cow's milk cream (21% energy) (HFLCD-SFAs), refined olive oil (21% energy) (HFLCD-MUFAs) or refined olive oil (20% energy) plus EPA+DHA (1% energy) (HFLCD-PUFAs). All the diets contained equal proportion of protein (19.5% energy) and carbohydrate was used to adjust the total energy content.

After weaning, mice were randomly allocated into 4 groups (n = 10 per group) as follows: (1) group that received LFLCD; (2) group that received HFLCD-SFAs; (3) group that received HFLCD-MUFAs; and (4) group that received HFLCD-PUFAs. The four groups received NA (1%, w/v) in the drinking water. Body weight, food, and water intake were daily evaluated. Sacrifice of all animals was carried out within the animal facilities (Instituto de Biomedicina de Sevilla, IBiS), at the beginning of the

light cycle and after 10 h of food deprivation. Animals were euthanized with an overdose of pentobarbital (1:10 in PBS, 150 mg/kg body weight). Serum samples were obtained from blood by centrifugation at 4000 rpm for 15 min at 4 ºC. Total cholesterol (TC) and triglycerides (TGs) were assessed by colorimetric assay kit (Bio-Science-Medical, Madrid, Spain). All animal protocols received appropriate institutional approval (Animal care and Use Committee of the University of Seville) and were performed according to the official rules formulated in the Spanish law on the care and use of experimental animals (UE Directive of 2010: 2010/63/UE; RD 53/2013).

Isolation of bone marrow cells and differentiation into BMDMs

Cells from bone marrow were isolated and pooled for each group of mice. Femora and tibiae were aseptically removed and dissected free of adherent soft tissue. The bone ends were cut, and the marrow cavity was flushed out into a petri dish by slowly injecting ice-cold PBS solution at one end of the bone using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain a single cell suspension. Cells were washed and depleted of red blood cells using a hypotonic lysis buffer.[9] After washing twice with PBS, the cells were cultured and differentiated into BMDMs for 6 days at 37 ºC under a humidified atmosphere containing 5% CO_2 in 12-well dishes containing RPMI 1640 medium (containing 50 units/mL penicillin G, 50 μg/mL streptomycin, 2 mM glutamine) plus 10% heat-inactivated FBS and 20 ng/mL of murine M-CSF. As controls for M1 and M2 polarisation, BMDMs from animals fed with LFLCD were further exposed to LPS (100 ng/mL) plus IFNγ (20 ng/mL) and to IL-4 (20 ng/mL), respectively, for additional 24 h.[10]

Measurement of the lipid content in lipid-laden BMDMs

BMDMs were washed twice with PBS and scraped in 400 μL of 0.9% NaCl. After sonication, the protein content of the lysate was measured using the Bradford protein assay (BioRad Laboratories, Madrid, Spain). Cellular lipids were extracted using hexane/isopropanol (3:2, v/v). The content of TC and TGs was measured using the aforementioned assay kits.[9]

RNA isolation and RT-qPCR analysis

Total RNA extraction from BMDMs was carried out using Trisure Reagent (Bioline, London, UK). RNA quality was assessed by A_{260}/A_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA (1 μg) was subjected to reverse transcription (iScript, BioRad). An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (BioRad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (BioRad) containing the primer pairs for the corresponding gene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The sequence and information for the primers are shown in **Supplemental Table 1**. All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to the content of housekeeping gene and expressed as percentage of control.

Statistical analysis

All values in the figures and text are expressed as arithmetic means ± SD. For ex vivo studies, serum and BMDMs isolated from the animals were pooled into four distinct

fractions. Experiments were carried out in triplicate. Data were evaluated with Graph Pad Prism Version 5.01 software. The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA); using Tukey multiple comparisons test as post hoc test. *P* values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

We have analysed the effects on macrophage plasticity of a LFLCD and HFLCDs of different FA compositions (SFAs, MUFAs, and MUFAs + omega-3 PUFAs) in NA-treated $Lep^{ob/ob}LDLR^{-/-}$mice at the end of an 8-week diet period. Mean daily food intake (LFLCD, 4.07 ± 0.32; HFLCD-SFAs, 4.15 ± 0.61; HFLCD-MUFAs, 4.01 ± 0.47; HFLCD-PUFAs, 4.17 ± 0.41 g/mouse) and water intake (LFLCD, 6.28 ± 0.83; HFLCD-SFAs, 6.35 ± 1.01; HFLCD-MUFAs, 6.12 ± 0.77; HFLCD-PUFAs, 6.11 ± 0.81 mL/mouse) were not different among the four diet groups. However, significant differences were observed from the second week for body weight and final body weight gain in animals subjected to the different diets (HFLCD-SFAs > HFLCD-MUFAs = HFLCD-PUFAs > LFLCD) (**Figs. 1A** and **1B**). These results support the notion of poor utilization of dietary SFAs for energy and their preferential storage in WAT.[11] In agreement with previous studies,[8] HFLCDs induced circulating lipid abnormalities in $Lep^{ob/ob}LDLR^{-/-}$mice (**Figs. 1C** and **1D**); additionally, we observed that serum TC levels were not different between HFLCD groups but higher than the LFLCD group (**Fig. 1C**), whereas serum TG levels were as follows: HFLCD-SFAs > HFLCD-MUFAs = HFLCD-PUFAs > LFLCD (**Fig. 1D**). Similar patterns were observed for intracellular TC (**Fig. 2A**) and TGs (**Fig. 2B**) in BMDMs obtained from the different diet groups. TG storage has been previously shown to increase in murine macrophages upon activation in vitro,[12] an effect that is blunted in the presence of

NA.[13] Our study further expands this knowledge and is suggestive of an in vivo predisposition of macrophages for lipid accumulation in a dietary FA-dependent manner in a setting of NA treatment.

Lipid accumulation in macrophages contributes to foam cell formation, which is associated with chronic subclinical inflammation and constitutes a hallmark of early atherosclerosis.[14] It was noteworthy that BMDMs from animals fed with HFLCD-SFAs had higher mRNA levels of pro-inflammatory M1 phenotypic markers CCR7 and iNOs[5] than BMDMs from animals fed with HFLCD-MUFAs or HFLCD-PUFAs, with no differences between both diets (**Figs. 3A** and **3B**). However, gene expression of anti-inflammatory M2 phenotypic markers MRC1 and Arg-1 (Cita) was upregulated in BMDMs from animals fed with HFLCD-MUFAs and HFLCD-PUFAs but not with HFLCD-SFAs (**Figs. 3C** and **3D**). There was also a decrease in the transcriptional activity of pro-inflammatory cytokine genes TNF-α, IL-1β, IL-6 and MCP-1 in BMDMs from animals fed with HFLCD-MUFAs and HFLCD-PUFAs when compared to BMDMs from animals fed with HFLCD-SFAs (**Figs. 3E-3H**). These findings are in accordance with previous studies on the potential of SFAs to promote inflammatory signals[15] in contrast to unsaturated Fas[6,16] and demonstrate divergent effects for SFAs with regard to MUFAs or MUFAs plus omega-3 long-chain PUFAs associated with NA in driving macrophage plasticity. BMDMs from animals fed with HFLCD-SFAs resembled M1 macrophages, whereas BMDMs from animals fed with HFLCD-MUFAs or HFLCD-PUFAs resulted in a macrophage phenotype close to that of M2. It is tempting to speculate that the nature of dietary fats could be behind the unexpected failure of some human trials to demonstrate the beneficial effects of NA in reducing cardiovascular disease (CVD) events.[17-19] Future studies are needed to determine the role of the diet in high-risk CVD patients

refractory to NA therapy. Taken together, our observations suggest that the combination of NA and dietary sources of MUFAs (e.g. olive oil) and of EPA and DHA may be useful for the management of chronic activation of the innate immune system in MetS.

Conflicts of interest

The authors state no conflict of interest.

Acknowledgements

This study was supported by research Grant AGL2011-29008 (Spanish Ministry of Science and Innovation, MICINN). S.M. has the benefit of a FPI fellowship (BES-2012-056104) of MICINN. B.B. and S.L. acknowledges financial support from "V Own Research Plan" (University of Seville) and the Spanish Research Council (CSIC)/Juan de la Cierva, respectively.

ABBREVIATIONS

BMDM: Bone marrow-derived macrophage

DHA: Docosahexaenoic acid

EPA: Eicosapentaenoic acid

FA: Fatty acid

HFLCD: High-fat low-cholesterol diet

IFN: Interferon

IL: Interleukin

LDLR: Low density lipoprotein receptor

LFLCD: Low-fat low-cholesterol diet

LPS: Lipopolysaccharide

M-CSF: Macrophage colony stimulating factor

MetS: Metabolic syndrome

MUFA: Monounsaturated fatty acid

NA: Niacin

NAD: Nicotinamide adenine dinucleotide

PBS: Phosphate buffered saline

PUFA: Polyunsaturated fatty acid

SFA: Saturated fatty acid

TC: Total Cholesterol

TG: Triglyceride

WAT: White adipose tissue

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Figure Legends

Figure 1. Body weight evolution (A), body weight gain (B), serum total cholesterol (C) and triglycerides (D) in male $Lep^{ob/ob}LDLR^{-/-}$ mice subjected to low-fat (LFLCD) and high-fat (HFLCD-SFAs, HFLCD-MUFAs and HFLCD-PUFAs) diets and NA (1%, w/v) in the drinking water for 8 weeks. Values are presented as means ± SD (n = 10). Different letters denote statistical differences (P <0.05).

Figure 2. Accumulation of total cholesterol (A) and triglycerides (B) in BMDMs from male $Lep^{ob/ob}LDLR^{-/-}$ mice subjected to low-fat (LFLCD) and high-fat (HFLCD-SFAs, HFLCD-MUFAs and HFLCD-PUFAs) diets and NA (1%, w/v) in the drinking water for 8 weeks. Values are presented as means ± SD (n = 10). Different letters denote statistical differences (P <0.05).

Figure 3. Relative mRNA levels of CCR7 (A), iNOs (B), MRC1 (C), Arg-1 (D), TNF-α (E), IL-1β (F), IL-6 (G) and MCP-1 (H) in BMDMs from male $Lep^{ob/ob}LDLR^{-/-}$ mice subjected to low-fat (LFLCD) and high-fat [HFLCD-SFAs (black bars), HFLCD-MUFAs (dark grey bars) and HFLCD-PUFAs (light grey bars)] diets and NA (1%, w/v) in the drinking water for 8 weeks. M1 and M2 control macrophages (white bars) were generated as indicated in the Materials and methods section. Values are presented as means ± SD (n = 10). Different letters denote statistical differences (P <0.05).

Table 1. Fatty acid composition of dietary fats.

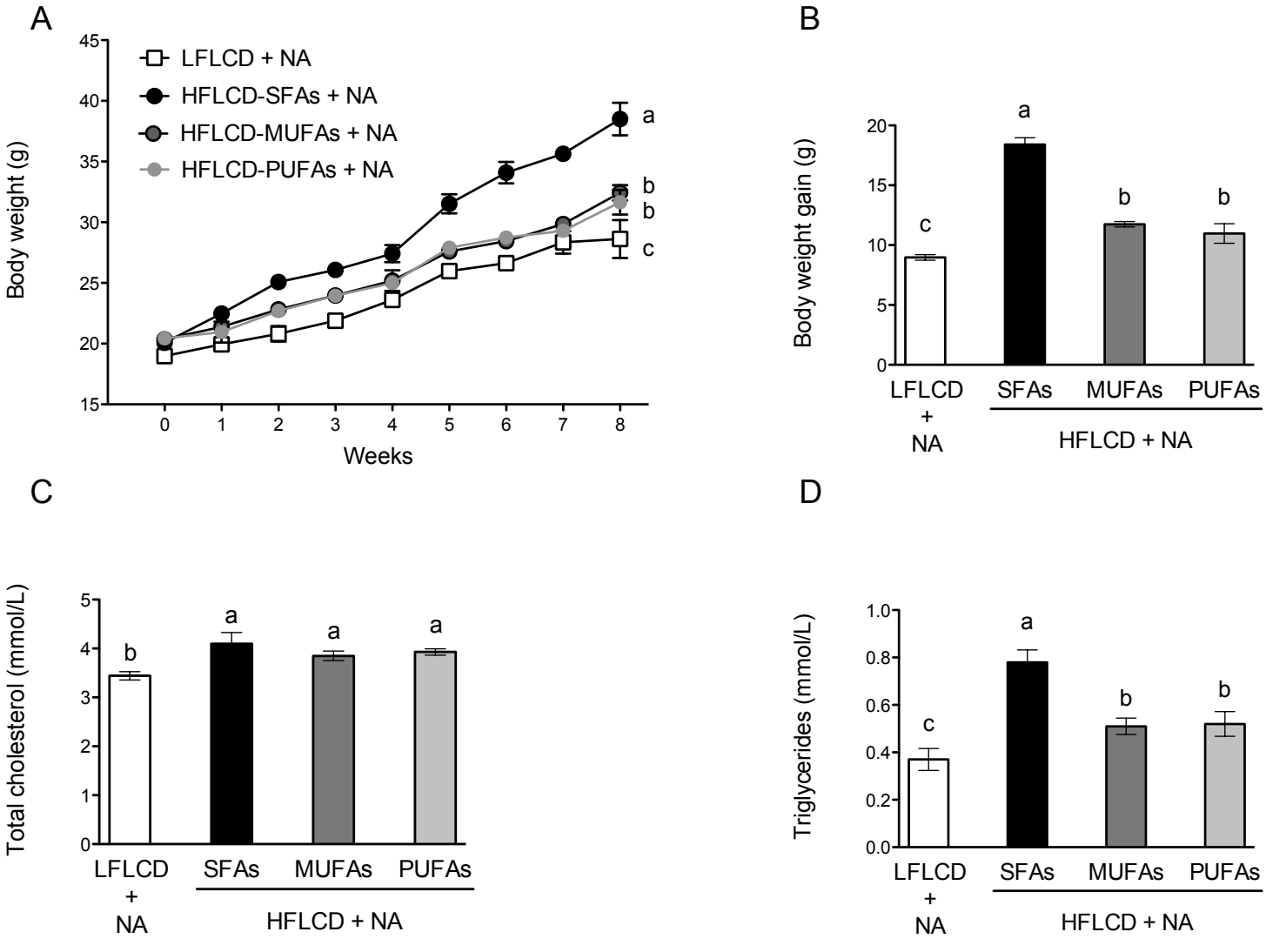
	Cow's milk cream	Refined olive oil	Refined olive oil plus EPA+DHA
Fatty acid		**g/100 g of fatty acid**	
10:0, capric	2.47 ± 0.13	-	-
12:0, lauric	3.09 ± 0.42	-	-
14:0, myristic	10.87 ± 0.91	-	-
16:0, palmitic	35.54 ± 0.82	20.41 ± 0.89	20.52 ± 0.64
16:1(n-7), palmitoleic	3.60 ± 0.32	0.97 ± 0.17	0.82 ± 0.12
18:0, stearic	11.49 ± 0.75	5.70 ± 0.11	4.49 ± 0.36
18:1(n-9), oleic	25.33 ± 0.71	61.86 ± 1.23	61.54 ± 0.97
18:2(n-6), linoleic	4.27 ± 0.82	7.97 ± 0.65	8.04 ± 0.53
18:3(n-3), α-linolenic	0.39 ± 0.05	1.04 ± 0.13	0.94 ± 0.03
20:5(n-3), eicosapentaenoic	-	-	0.92 ± 0.09
22:6(n-3), docosahexaenoic	-	-	0.72 ± 0.10
Others	2.95 ± 1.72	2.05 ± 1.08	2.01 ± 0.88
Total SFAs	63.46 ± 1.86^{a}	26.11 ± 1.03^{b}	25.01 ± 0.85^{b}
Total MUFAs	28.93 ± 0.83^{b}	62.83 ± 1.35^{a}	62.36 ± 1.03^{a}
Total PUFAs	4.66 ± 0.79^{c}	9.01 ± 0.73^{b}	10.62 ± 0.68^{a}

Values are means ± SD, n = 3. Different superscript letters denote mean values in a row significantly different ($P < 0.05$).

Supplementa Table 1. Sequences of RT-qPCR primers for gene expression analysis.

Target	No. *GenBank*	Direction	Sequence (5' --> 3')
GAPDH	NM_001289726	Forward	AACTTTGGCATTGTGGAAGG
		Reverse	ACACATTGGGGGTAGGAACA
CCR7	NM_001301713	Forward	CCAGGCACGCAACTTTGAG
		Reverse	ACTACCACCACGGCAATGATG
iNOs	NM_010927	Forward	CACCTTGGAGTTCACCCAGT
		Reverse	ACCACTCGTACTTGGGATGC
MRC1	NM_008625.2	Forward	ATGCCAAGTGGGAAAATCTG
		Reverse	TGTAGCAGTGGCCTGCATAG
Arg-1	NM_007482	Forward	GTGAAGAACCCACGGTCTGT
		Reverse	CTGGTTGTCAGGGGAGTGTT
TNF-α	NM_001278601	Forward	AGCCCCCAGTCTGTATCCTT
		Reverse	CTCCCTTTGCAGAACTCAGG
IL-1β	NM_008361	Forward	GCCCATCCTCTGTGACTCAT
		Reverse	AGGCCACAGGTATTTTGTCG
IL-6	NM_031168	Forward	AGTTGCCTTCTTGGGACTGA
		Reverse	TCCACGATTTCCCAGAGAAC
MCP-1	NM_011331	Forward	AGGTCCCTGTCATGCTTCTG
		Reverse	TCTGGACCCATTCCTTCTTG

Figure 1


A
Body weight (g)
LFLCD + NA
HFLCD-SFAs + NA
HFLCD-MUFAs + NA
HFLCD-PUFAs + NA
Weeks
a
b
b
c
B
Body weight gain (g)
LFLCD + NA
SFAs
MUFAs
PUFAs
HFLCD + NA
C
Total cholesterol (mmol/L)
D
Triglycerides (mmol/L)

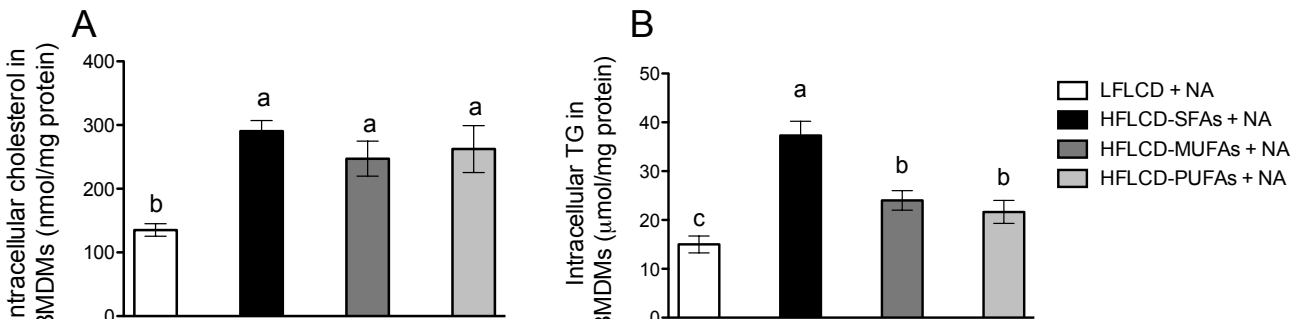
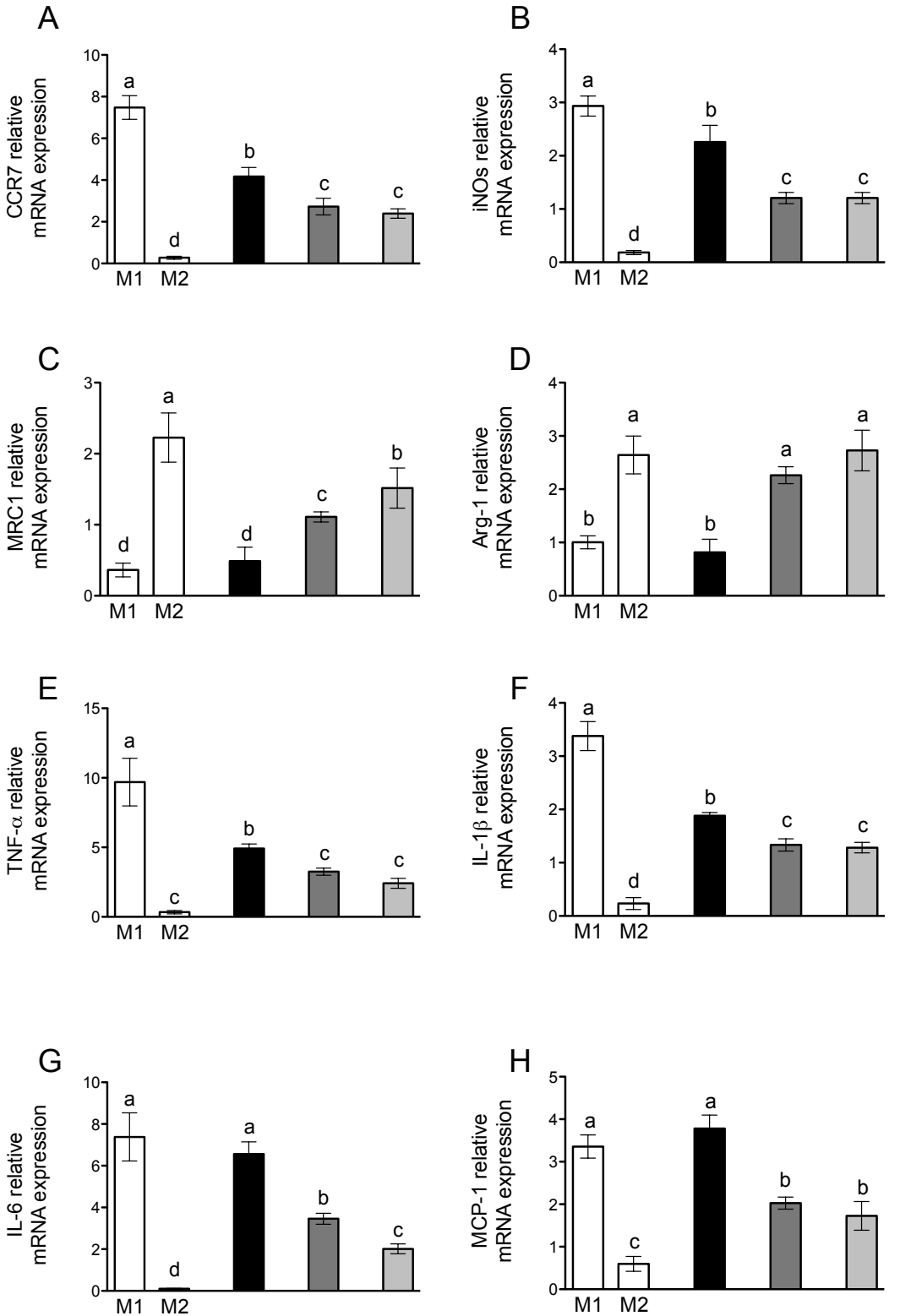

A
Intracellular cholesterol in BMDMs (nmol/mg protein)
400
300
200
100
0
b
a
a
a
B
Intracellular TG in BMDMs (μmol/mg protein)
50
40
30
20
10
0
c
a
b
b
LFLCD + NA
HFLCD-SFAs + NA
HFLCD-MUFAs + NA
HFLCD-PUFAs + NA

Figure 3


A
CCR7 relative mRNA expression
10
8
6
4
2
0
a
d
b
c
c
M1
M2
B
iNOs relative mRNA expression
4
3
2
1
0
a
d
b
c
c
M1
M2
C
MRC1 relative mRNA expression
3
2
1
0
d
a
d
c
b
M1
M2
D
Arg-1 relative mRNA expression
4
3
2
1
0
b
a
b
a
a
M1
M2
E
TNF-α relative mRNA expression
15
10
5
0
a
c
b
c
c
M1
M2
F
IL-1β relative mRNA expression
4
3
2
1
0
a
d
b
c
c
M1
M2
G
IL-6 relative mRNA expression
10
8
6
4
2
0
a
d
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b
c
M1
M2
H
MCP-1 relative mRNA expression
5
4
3
2
1
0
a
c
a
b
b
M1
M2

Niacin and olive oil promote the skewing to M2 phenotype in bone marrow-derived macrophages of mice with metabolic syndrome