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Original article

# Milk fat globule membrane modulates inflammatory pathways in human monocytes: A crossover human intervention study

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SUMMARY

*Background:* Intake of high-fat foods raises postprandial plasma triglycerides and inflammatory markers, which may depend on the type of fat ingested. Dairy products are commonly consumed, but not much is known about the impact of milk fat and the milk fat globule membrane on postprandial inflammation. Here, we aimed to study the effect of milk fat with and without milk fat globule membrane and a vegetable fat blend on post-prandial inflammation, with a focus on blood monocyte gene expression.
*Methods:* We performed a randomized, double-blind cross-over trial in 37 middle-aged healthy male and female volunteers (BMI 22–27 kg/m$^2$). The participants consumed a meal shake containing 95.5 g of fat consisting of either a vegetable fat blend (VEGE), anhydrous milk fat (AMF, without milk fat globule membrane), or cream (CREAM, containing milk fat globule membrane). Blood monocytes were collected at 0 h and 6 h postprandially and used for bulk RNA sequencing and ex vivo stimulation with LPS.
*Results:* Consumption of all three shakes significantly decreased the percentage of classical monocytes and increased the percentages of intermediate monocytes and non-classical monocytes. No differences in these measures were observed between shakes. Using a threshold of $p < 0.01$, 787 genes were differentially regulated postprandially between the three shakes. 89 genes were differentially regulated postprandially between AMF and VEGE, 373 genes between AMF and CREAM, and 667 genes between VEGE and CREAM, indicating that the effect of CREAM on monocyte gene expression was distinct from AMF and VEGE. Pathway analyses showed that VEGE significantly increased the expression of genes involved in inflammatory pathways, whereas this was less pronounced after AMF and not observed after CREAM. In addition, CREAM significantly down-regulated the expression of genes involved in energy metabolism-related pathways, such as glycolysis, TCA cycle, and oxidative phosphorylation, as well as HIF-1 signaling.
*Conclusion:* Compared to the consumption of an anhydrous milk fat without milk fat globule membrane and a vegetable fat blend, the consumption of cream with milk fat globule membrane downregulated inflammatory pathways in blood monocytes, thus suggesting a potential inflammation inhibitory effect of milk fat globule membrane.


## 1. Introduction

Triglycerides are a major source of energy in the human diet. After a meal, dietary triglycerides are hydrolyzed in the small intestine into fatty acids and monoglycerides. After uptake into enterocytes, the fatty acids and monoglycerides are re-converted into triglycerides, which are packaged into large emulsion particles called chylomicrons. The chylomicrons are secreted into the lacteals and arrive in the blood after passage through the splanchnic lymphatic system. Most of the chylomicrons are processed in the adipose tissue, heart, and skeletal muscle, where the triglycerides are hydrolyzed by the enzyme lipoprotein lipase (LPL). Elevated levels of triglycerides after a meal, referred to as postprandial lipemia, are associated with an increased risk of cardiovascular disease [1,2]. As a consequence, major efforts are

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undertaken to try to develop strategies aimed at attenuating the postprandial triglyceride response, for instance, by enhancing LPL-mediated lipolysis [3].

In addition to lipemia, the postprandial state is associated with inflammation. This postprandial inflammatory response is characterized by increased plasma levels of inflammatory markers, such as IL-6, TNFα, VCAM1, ICAM1, and endotoxins [4–8], as well as altered inflammatory gene expression in peripheral blood mononuclear cells (PBMCs) and monocytes [9–11]. It is thought that postprandial inflammation is closely connected to lipemia, possibly because of the direct activation of immune cells by triglyceride-rich lipoproteins [12]. Persistently increased postprandial inflammation may potentially contribute to a state of chronic low-grade inflammation, which is a common feature of cardiometabolic diseases [13].

An important fat source in the Western diet is dairy. For example, in the Netherlands, nearly 20% of the total dietary fat intake and nearly 33% of the intake of saturated fatty acids is derived from dairy [14]. Besides being rich in saturated fatty acids, milk fat is characterized by the presence of trans, branched- and odd-chain fatty acids and by a high content of butyrate. Additionally, milk fat is unique in that palmitate is highly enriched in the *sn*-2 position, whereas in common sources of vegetable fat, palmitate is highly enriched in the *sn*1,3 position. Another special property of milk fat is that it contains so-called milk fat globule membranes (MFGM). MFGM form the perimeter of the milk fat globules and are enriched with glycerophospholipids and sphingolipids, especially sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine. Other components of the MFGM include proteins, cholesterol, and other lipid species. Although several studies have pointed to the potential positive health properties of MFGM in a variety of clinical contexts, including insulin sensitivity and elevated plasma lipids, our overall understanding of the potential health effects of MFGM is still very limited [15].

Previous studies have shown that the acute postprandial inflammatory response may depend on the type of fat ingested. We previously observed that saturated and mono-unsaturated fatty acids differentially affected gene expression in human PBMCs [9,10]. Specifically, saturated fatty acids (SFA) decreased the expression of cholesterol biosynthesis and uptake genes and increased cholesterol efflux genes, whereas mono-unsaturated fatty acids (MUFA) increased inflammatory genes and PPARα targets involved in β-oxidation [9]. Similarly, dietary fatty acid composition substantially impacted the postprandial changes in the PBMC proteome [16]. Concerning milk fat, it was observed that fermented dairy products, especially cheese, induced a less inflammatory postprandial PBMC gene expression response than non-fermented dairy products [17]. However, a direct comparison between milk fat and vegetable fat, as well as an investigation of the specific role of MFGM, has yet to be performed.

PBMCs consist of a variety of different cell types, including T lymphocytes, B lymphocytes, and monocytes. Monocytes are innate immune cells that circulate in the blood and differentiate into macrophages after entry into tissues. After a high-fat meal, monocytes are exposed to postprandial lipemia characterized by high levels of very low-density lipoproteins (VLDL) and chylomicrons in the circulation. These triglyceride-rich lipoproteins may be taken up via caveolin-mediated endocytosis, leading to the time-dependent accumulation of lipids in monocytes after a high-fat meal [18,19]. This postprandial increase in monocyte lipid uptake and storage is likely associated with changes in (inflammatory) gene expression. Interestingly, however, the postprandial impact of a high-fat meal specifically on monocyte gene expression has not yet been investigated. In addition, to what extent these effects may differ between different fat sources and may be influenced by MFGM is unknown.

Here, we used RNAseq to compare the postprandial effect of the consumption of a high-fat meal containing cream (containing MFGM), anhydrous milk fat (lacking MFGM), or a vegetable fat blend on monocyte gene expression in healthy volunteers (BMI 22–27 kg/m$^2$).

## 2. Materials and methods

### 2.1. Study design and subjects

The study was conducted at Wageningen University, the Netherlands, from 07-01-2020 until 10-03-2020. The experimental protocol and procedures were approved by the Medical Ethical Committee of Wageningen University and were in accordance with the Helsinki Declaration of 1975 as revised in 1983. The study was registered at ClinicalTrials.gov with identifier NCT04178681 'Postprandial Effects of Milk Fats (POEMI)'. The study was a randomized, double-blind crossover intervention trial and aimed at comparing the postprandial effects of formula vegetable fat (VEGE), anhydrous milk fat (AMF), and cream (CREAM) on the human immune response. 59 healthy participants were screened for the trial based on the criteria: 40–70 years (age) and 22–27 kg/m$^2$ (BMI). Based on the power calculation to detect a significant effect on a panel of fourteen inflammatory genes to reach an effect size range from 0.089 to 0.283 with a power of 80% and a two-sided alpha of 0.0012 or a panel of eight cytokines including IL-8, to detect a treatment difference of 0.37 pg/mL, with a power of 80% and a two-sided alpha of 0.0021, and considering a 10% drop out rate tolerance, in the end, 40 participants were included. Both men and women were randomly included in the study. 40 participants were given three different shakes on different days. At least one week for washout between two interventions. On the test day, subjects arrived fasted, and the concentration of C-reactive protein (CRP) in the blood was determined via a finger prick using a QuickRead CRP test (Orion Diagnostica Oy) before any blood was collected. When the CRP concentration was below 10.0 mg/L, participants were qualified for the study, and a catheter cannula was inserted in an antecubital vein. After a 30-min rest, fasted blood was drawn into EDTA-containing tubes to establish baseline (t0) values for the parameters of interest. Subjects were then asked to consume a shake within 10 min. Blood was drawn again every additional hour post-shake, with the last blood collection taking place 8 h after consumption of the shake. In addition, subjects were advised to drink water regularly. Each subject consumed all three shakes in a randomized order.

### 2.2. Intervention diets

The three intervention fat shakes were supplied by Friesland-Campina. Shake VEGE is based on the lipid formula used in infant formula. To prepare the shake, 118 g powder was mixed with 500 mL skim milk for the VEGE shake and AMF shake, and 461 mL skim milk plus 39 mL water for the CREAM shake. Except for the presence of MFGM, shake AMF and shake CREAM contain the same milk-sourced lipids. All shakes contained 95 g of fat, 21 g of protein, and about 42 g of carbohydrates and provided around 4600 kJ (Table 2).

### 2.3. Identification of monocyte subsets in human whole blood

Whole blood from each subject and at each of the two time points (t = 0 and t = 6) was divided into two aliquots of 100 μL. One

aliquot was stained with antibodies, and the other half was left unstained to determine the levels of autofluorescence. Fluorescently tagged monoclonal antibodies were obtained from BD Biosciences (CD56-PE and CD16-FITC) and Beckman Coulter (CD14-ECD, HLA-DR-PeCy5, CD45-PeCy7, and CD3-PE). The staining procedure was as follows: first, whole blood was incubated with the amount of fluorescently tagged monoclonal antibody as specified by the manufacturer on ice for 20 min. After lysing the red blood cells using FACS Lysing Solution (BD Biosciences), samples were incubated for 15 min at room temperature and protected from light. We then centrifuged the samples for 5 min at 150 × *g* at room temperature and subsequently removed the supernatant. The pellet was immediately resuspended in 250 μL of FACS buffer (1% BSA in PBS, 0.22 μm filtered). All samples were analyzed on an FC500 flow cytometer (Beckman Coulter) at a medium flow rate. To identify classical, intermediate, and non-classical monocytes in whole blood based on CD14 and CD16 expression, a gating strategy described by Mukherjee et al. [20] was applied.

### 2.4. Human monocyte isolation

8 ml of blood was collected at t = 0 h and t = 6 h using a CPT tube and centrifuging at room temperature for 20 min at 1800 RCF after mixing by inverting tubes. After centrifugation, the white cloudy layer was collected for further PBMC purification by washing with cold PBS three times.

Monocytes were isolated by MojoSort CD14 negative selection kits (Biolegend, California, United States) and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were resuspended in 100 μL (per $10^7$ PBMCs; the same below) MojoSort buffer. Then, 5 μL Human TruStain FcX™ solution was added to block Fc receptor and incubated at room temperature for 10 min. After adding 10 μL of the Biotin-Antibody Cocktail, the cell solution was incubated on ice for 15 min. Thereafter, 10 μL of Streptavidin Nanobeads was added, and incubation was continued on ice for 15 min. After washing using MojoSort™ Buffer, the cells were separated by LS columns using a Miltenyi Quadro MACS Separator, and the flow through containing monocytes was collected and centrifuged.

### 2.5. RNA isolation and transcriptome analysis

At least 500,000 cells were collected from each subject for every intervention and both time points (t = 0 and t = 6) for total RNA isolation using RNeasy Micro Kit (Qiagen, Hilden, Germany). The quality of the RNA was determined using Agilent Bioanalyzer. RNA samples with a RIN value above 8 were included, as were a small number of samples with a lower RIN value but with relatively non-degraded 28S and 18S peaks. Transcriptome analysis by RNA sequencing was performed by BGI Company Limited (Copenhagen, Denmark) following a standard protocol. In brief, mRNA molecules were purified from total RNA using oligo(dT)-attached magnetic beads and fragmented into small pieces using fragmentation reagent after reaction for a certain period at the proper temperature. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by second-strand cDNA synthesis. The synthesized cDNA was subjected to end-repair and then was 3′ adenylated. Adapters were ligated to the ends of these 3′ adenylated cDNA fragments. PCR products were purified with Ampure XP Beads (AGENCOURT) and dissolved in EB solution. The library was validated on the Agilent Technologies 2100 bioanalyzer. The double-stranded PCR products were heat-denatured and circularized by the splint oligo sequence. The single-strand circle DNA was formatted as the final library. The library was amplified with phi29 to make DNA nanoballs with more than 300 copies of one molecule. The DNB nanoballs were loaded into the patterned nanoarray and pair-end 150 base reads were generated by sequencing by synthesis. The RNAseq reads were then used to quantify transcript abundances. To this end, the tool *Salmon* was used to map the reads to the GRCh38.p13 human genome assembly-based transcriptome sequences as annotated by the GENCODE consortium (release 37).

### 2.6. Ex vivo monocytes culture and cytokine measurements

500,000 monocytes were seeded in 24 well plates and cultured in RPMI medium supplied with 2 mM Glutamax, 1% penicillin/streptomycin, and 1 mM Pyruvate. After a short incubation for 1 h to get monocytes adherent, cells were stimulated with 10 ng/mL LPS for 24 h. The culture medium was collected afterward for cytokine analysis according to the manufacturer's protocol.

### 2.7. Data analysis

The obtained transcript abundance estimates and lengths were imported in R using the package *tximport*, scaled by average transcript length and library size, and summarized on the gene level. Such scaling corrects for bias due to correlation across samples and transcript length and has been reported to improve the accuracy of differential gene expression analysis [21]. Differential gene expression was determined using the package *limma* [22] utilizing the obtained scaled gene-level counts. Briefly, before statistical analyses, nonspecific filtering of the count table was performed to increase detection power, based on the requirement that a gene should have an expression level greater than 10 counts, i.e. ~0.45 count per million reads (cpm) mapped, for at least 3 libraries across all 9 samples. Differences in library size were adjusted by the trimmed mean of M-values normalization method [23], implemented in the package *edgeR* [24]. Counts were transformed to log2(cpm) values and associated precision weights and entered into the *limma* analysis pipeline [25]. Differentially expressed genes were identified by using generalized linear models that incorporate empirical Bayesian methods to shrink the standard errors towards a common value, thereby improving testing power [26]. ANOVA-like F-test was performed to determine the significance.

For gene set enrichment analysis (GSEA), 344 KEGG gene sets were considered. After filtering out the sets that contain genes that

**Table 1**
Characteristics of the 40 included participants in this study.

| Characteristic | |
|---|---|
| Gender (m/f) | 9/31 |
| Age (years) | 57.3 ± 8.0 |
| Weight (kg) | 71.5 ± 8.5 |
| Height (cm) | 172.2 ± 7.7 |
| BMI (kg/m$^2$) | 24.1 ± 1.7 |
| Waist circumference (cm) | |
| Men | 92.2 ± 5.9 |
| Women | 81.8 ± 6.2 |
| Hip circumference (cm) | |
| Men | 102.6 ± 4.6 |
| Women | 102.2 ± 6.0 |
| WHR | |
| Men | 0.90 ± 0.05 |
| Women | 0.80 ± 0.05 |
| Hb (mmol/L) | 8.4 ± 0.6 |
| ALAT (μL) | 24.7 ± 11.8 |
| ASAT (μL) | 21.5 ± 7.3 |
| Creatinine (μmol/L) | 71.6 ± 11.1 |

Data are presented as mean ± standard deviation. Abbreviations: alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), body mass index (BMI), hemoglobin (Hb), waist-hip ratio (WHR).

are not measured and below the minimum size of 15 genes or above the maximum size of 500 genes, 308 gene sets were kept.

Ingenuity Pathway Analysis (IPA) is a software application that allows searches for targeted information on genes, proteins, chemicals, and drugs and the building of interactive models of experimental systems. The software is backed by the Ingenuity Knowledge Base of highly structured, detail-rich biological and chemical findings. To predict the upstream regulators of the differential modulations on the transcriptome of monocytes, IPA was performed. The genes differentially expressed with $p < 0.05$ in the comparisons were considered.

To analyze correlations between the transcriptional data and metabolomics dataset obtained from the same individuals using the Nightingale nuclear magnetic resonance spectroscopy (NMR) platform (Nightingale Health, Helsinki, Finland), regularized Canonical Correlation Analysis (rCCA) was performed using the *mixOmics* R package (http://mixomics.org/cite-us/). Differentially expressed genes between any combination of two shakes (by ANOVA-like F-test) and metabolites were transformed to Δlog2 units between t = 0 and t = 6 for each individual and imported in R. Regularization penalties (λ1, λ2) were determined by shrinkage approach. rCCA was performed separately for the three shakes, after which the correlation matrices were extracted and plotted as clustered heatmaps. Correlation cut-off for plotting genes was set at > 0.5 with any metabolite in at least one of the three analyses.

## 3. Results

### 3.1. Participants and interventions

Of the 40 participants who entered this study, 37 completed the study, meaning that they completed all three interventions. One

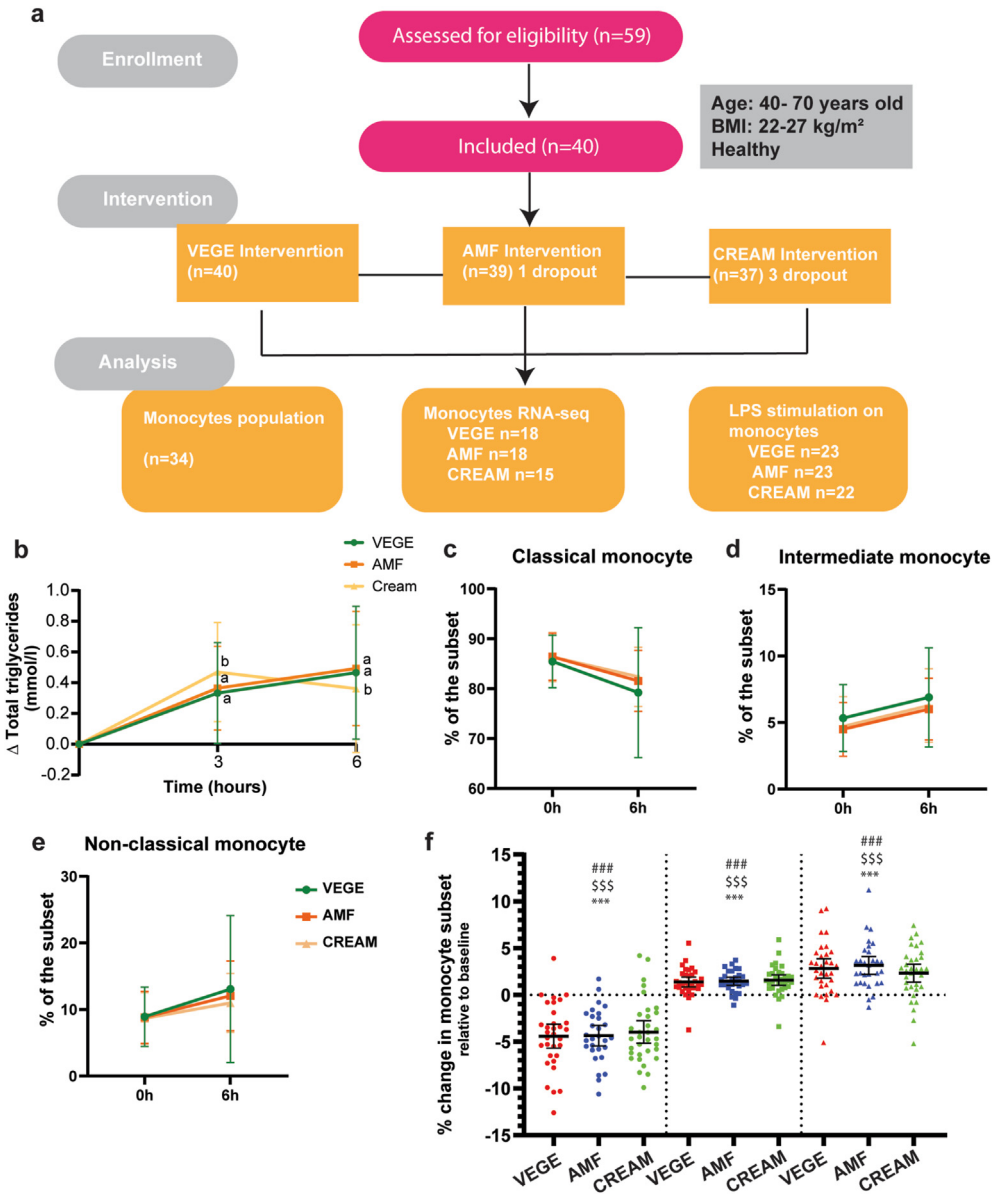


**Fig. 1.** Post-prandial effects of the three interventions. (a) Flow charts of study participants. (b) Serum triglycerides 3 h and 6 h after shake consumption. Shake*time effect in linear mixed models analysis (FDR p-value = 0.008). At t = 3 h, triglycerides were significantly different between VEGE and CREAM (p = 0.004), and AMF and CREAM (p = 0.029). At t = 6 h, triglycerides were significantly different between VEGE and CREAM (p = 0.005), and AMF and CREAM (p < 0.001). a, b, c indicate significant differences between shakes for shake*time effect at specific time points. (c–f) The percentage changes in monocyte population after shake consumption. (f) Left panel, classical monocytes; middle panel, intermediate monocytes; right panel, non-classical monocytes. #, $ and * indicate significant postprandial change. ###p < 0.001, $$$ p < 0.001 and ***p < 0.001.

participant dropped out after the first study visit because of not being willing to participate further, thereby missing the CREAM and AMF interventions. Two participants dropped out after the second study visit because they were unavailable to plan the last study day, thereby missing the CREAM intervention. The baseline characteristics of the participants are shown in Table 1. Monocytes were isolated from all participants for RNA extraction. RNA samples with sufficient quality RNA were selected for whole transcriptome analysis, which included 18 samples from the VEGE intervention, 18 samples from the AMF intervention, and 15 samples from the CREAM intervention. The flowchart of the inclusion of participants is shown in Fig. 1a.

### 3.2. Postprandial changes in monocyte subsets

The postprandial total triglycerides in blood were determined using the NIGHTINGALE platform. After 3 h, all interventions significantly increased blood total triglycerides (Fig. 1b). Interestingly, the CREAM intervention caused a significant rapid increase followed by a decrease in total postprandial triglyceride levels, compared to the other two interventions (Fig. 1b). Using flow cytometry, we studied monocyte phenotypes of 34 participants after consumption of the shakes. Classical monocytes were defined as $CD14^{+}/CD16^{-}$ cells, intermediate monocytes as $CD14^{+}/CD16^{+}$, and non-classical monocytes as $CD14^{dim}/CD16^{+}$. No significant differences were observed between the postprandial effects of the three interventions on monocyte phenotypes (Fig. 1c–e). Consumption of the three shakes led to a significant postprandial decrease in the percentage of classical monocytes and a significant increase in the percentages of intermediate monocytes and non-classical monocytes (Fig. 1f).

### 3.3. Consumption of CREAM differentially modulates the monocyte whole transcriptome compared to AMF and VEGE

The nutritional composition of the shakes, including the fatty acid composition, is depicted in Tables 2 and 3. The total amount of fat was the same across the shakes. However, the VEGE shake had a lower percentage of saturated fatty acids and a higher percentage of mono- and polyunsaturated fatty acids than the milk fat-based shakes, whose fatty acid compositions were highly similar. Specifically, saturated fatty acids (SFA) accounted for 41.3% of the total fatty acids in VEGE, 70.4% in AMF, and 71.2% in CREAM. VEGE was rich in both mono-unsaturated fatty acids (MUFA, 40.0%) and poly-unsaturated fatty acids (PUFA, 18.6%). Compared to VEGE, the percentage of MUFA and PUFA was much lower in AMF and CREAM at around 25.0% and 3.0%, respectively. The majority of PUFA in VEGE were omega-6 fatty acids, which accounted for 17.0% of the total fatty acids. By comparison, omega-6 fatty acids represented 1.8% of the total fatty acids in AMF and CREAM.

To establish the postprandial effect of the different shakes on gene expression in monocytes, whole transcriptome bulk RNAseq analysis was performed. After filtering, 12,770 protein-coding genes with substantial expression remained from a total of 60,232 transcripts (Fig. 2a). To establish the difference between shakes, an ANOVA-like F-test was performed to analyze the differential effect of the VEGE, AMF, and CREAM interventions on whole transcriptome gene expression. Using a threshold of $p < 0.01$, 787 genes were differentially regulated postprandially between the three shakes. In total, 89 genes were differentially regulated postprandially by the AMF intervention compared to the VEGE intervention, while 373 genes were differentially regulated by AMF compared to CREAM, and 667 genes were differentially regulated by VEGE compared to CREAM ($p < 0.01$) (Fig. 2a). These numbers indicate that the effects of the CREAM intervention on monocyte gene expression were distinct from the AMF intervention and VEGE intervention. A heatmap of the average change in expression of the genes differentially expressed between all shakes is shown in Fig. 2b.

To gain more insight into the specific biological pathways affected by the different interventions, we performed Gene Set Enrichment Analysis (GSEA). Many pathways were commonly

**Table 3**
Fatty acid composition of intervention shakes (mole percentage).

| | VEGE | AMF | CREAM |
|---|---|---|---|
| C4:0 | | 3.9 | 3.8 |
| C6:0 | <0.1 | 2.3 | 2.4 |
| C8:0 | 1.0 | 1.3 | 1.4 |
| C10:0 | 0.8 | 3.0 | 3.2 |
| C11:0 | <0.1 | 0.4 | 0.4 |
| C12:0 | 11.5 | 3.9 | 4.2 |
| C12:1w1cis | | 0.1 | 0.1 |
| C14:0 | 4.1 | 11.4 | 11.6 |
| C14:1w5cis | | 1.0 | 1.1 |
| C15:0 | <0.1 | 1.1 | 1.1 |
| C15:iso | | 0.2 | 0.2 |
| C15:aiso | | 0.4 | 0.4 |
| C16:0 | 19.8 | 30.4 | 31.4 |
| C16:iso | <0.1 | 0.2 | 0.2 |
| C16:1w7cis | 0.1 | 1.5 | 1.6 |
| C17:0 | <0.1 | 0.5 | 0.5 |
| C17:iso | | 0.4 | 0.3 |
| C17:aiso | <0.1 | 0.7 | 0.6 |
| C17:1w7cis | <0.1 | 0.2 | 0.2 |
| C18:0 | 3.3 | 9.7 | 9.1 |
| C18:1 | 37.7 | 18.3 | 17.9 |
| C18:1w7tr | | 1.3 | 1.0 |
| C18:1w12tr | | 0.1 | 0.1 |
| C18:1w12cis | | 0.8 | 0.7 |
| C18:1w9tr | <0.1 | 0.2 | 0.2 |
| C18:1w9cis | 1.6 | 0.7 | 0.7 |
| C18:1w6cis | | 0.8 | 0.7 |
| C18:2w6cis | 16.9 | 1.3 | 1.4 |
| C18:2conj cis9t11 | | 0.5 | 0.4 |
| C18:3 | 1.6 | 0.5 | 0.4 |
| C20:1 | 0.4 | <0.1 | <0.1 |
| C22:6w3cis | <0.1 | <0.1 | <0.1 |
| **Summary** | | | |
| Saturated fatty acids | 41.3 | 70.4 | 71.2 |
| Monounsaturated fatty acids | 40.0 | 25.2 | 24.5 |
| Polyunsaturated fatty acids | 18.6 | 2.8 | 2.6 |
| Trans fatty acids | <0.1 | 1.9 | 1.7 |
| Omega-3 fatty acids | 1.6 | 0.7 | 0.6 |
| Omega-6 fatty acids | 17.0 | 1.8 | 1.8 |
| Sn-2 palmitate | 15 | 45 | 45 |

**Table 2**
Intervention shake contents.

| | Powder (118 g) | | | | Additional | | | | Total | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fat (g) | Protein (g) | Lactose (g) | Glucose (g) | Fat (g) | Protein (g) | Lactose (g) | Glucose (g) | Fat (g) | Protein (g) | Carbs (g) | Energy (kJ) |
| VEGE & AMF | 95.0 | 2.4 | 0.2 | 17.6 | 0.5 | 18.5 | 24.5 | 0.0 | 95.5 | 20.9 | 42.3 | 4607.9 |
| CREAM | 95.0 | 4.2 | 5.5 | 11.3 | 0.5 | 17.0 | 22.5 | 3.2 | 95.5 | 21.2 | 42.5 | 4616.4 |

VEGE and AMF were dissolved in 500 mL milk and CREAM was dissolved in 461 mL milk and 39 mL water (the specific calculation of nutrients is listed in “Additive”).

enriched by the VEGE and AMF interventions (Fig. 3a), while other pathways were uniquely suppressed by the CREAM intervention (Fig. 3b–c). In the between-shake comparisons, several differentially regulated pathways (FDR q < 0.25) were identified in the comparison of CREAM and VEGE (119 gene sets) and the comparison of CREAM and AMF (100 gene sets), whereas fewer gene sets were differentially regulated between AMF and VEGE (26 gene sets) (Fig. 3d). This indicates a similarity in the postprandial effects of AMF and VEGE on monocyte gene expression and a distinct effect of CREAM. By analyzing these pathways in the different categories defined by KEGG, we found that several energy metabolism-related gene sets were upregulated by VEGE, not regulated by AMF, and downregulated by CREAM (Fig. 4a). Gene sets related to environmental information processing and immune and metabolic pathways were specifically upregulated by VEGE or upregulated by VEGE and AMF, respectively (Fig. 4b & c).

### 3.4. Milk fat-based shakes caused a lower postprandial inflammatory response in monocytes than VEGE

A Cytoscape map was plotted to visualize the overlapping genes and highlight clusters (Fig. S1). We found that inflammation-related pathways (in cluster 1), such as the TNF signaling pathway and Toll-like receptor signaling pathway, were significantly upregulated by the VEGE shake but were not significantly altered by the AMF and CREAM shake. In line with this, a heatmap of the leading-edge genes showed an overall pattern of upregulation by VEGE, which was less pronounced or even opposite in response to the AMF and CREAM interventions, respectively (Fig. 5a). The genes belonging to the 'positive regulation of the inflammatory response' (GO:0050729) were induced by the VEGE intervention, showed a partly similar response to the AMF intervention, and were generally downregulated by the CREAM intervention (Fig. 5b).

To investigate the functional consequence of consumption of the different shakes on the monocytes, monocytes isolated at baseline and 6 h postprandially were incubated with LPS, followed by the measurement of the release of pro-inflammatory cytokines. The LPS-induced release of TNFα by monocytes was decreased after the consumption of all three shakes compared to baseline (Fig. 5c). However, no significant differences in the postprandial decrease in LPS-induced TNFα release were observed between the shakes. Additionally, no significant postprandial effect was observed on LPS-induced secretion of IL-6 for any of the shakes (Fig. 5d).

### 3.5. Milk fat globule membrane suppresses energy metabolism-related signaling pathways

As shown in Figs. 4a and S1 (cluster 2), the CREAM intervention downregulated energy metabolism pathways in monocytes, including glycolysis, TCA cycle, and oxidative phosphorylation, the latter suggesting reduced mitochondrial activity. This is supported by the individual changes in the genes involved in these pathways (Fig. 6a). We also evaluated the regulation of genes involved in ATP-coupled electron transportation (GO:0042773) (Fig. 6b) and oxidative stress-related pathway (Fig. 6c), suggesting a decreased mitochondrial activity in response to CREAM but not the other interventions. Interestingly, a significant downregulation of the *LDHA* and *LDHB* genes encoding lactate dehydrogenase was observed after CREAM consumption (Fig. 6d), which is in line with the observed downregulation of the TCA cycle [27,28].

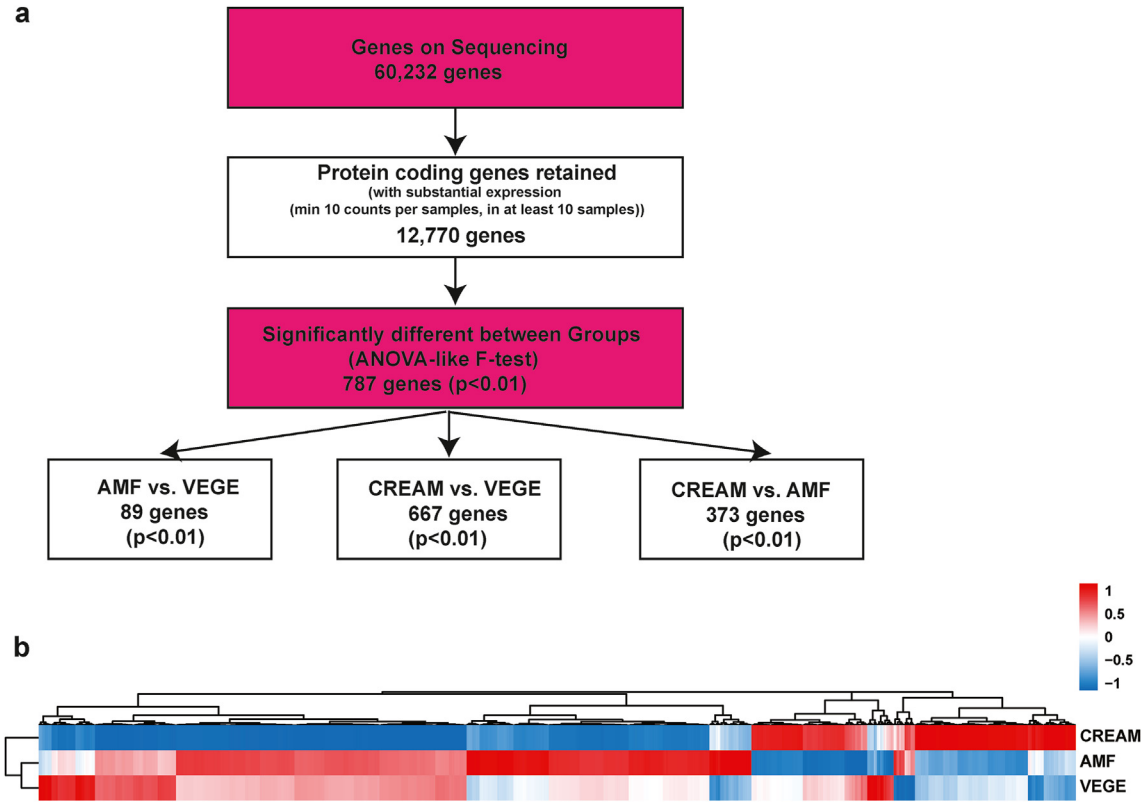


**Fig. 2.** Distinct effects of VEGE, AMF, and CREAM intervention on the whole transcriptome of monocytes. (a) The number of genes differentially changed between interventions (ANOVA-like F-test). (b) The heatmap of genes significantly changed in any between-shake comparisons (ANOVA-like F-test) and significantly regulated by any shake (Signal Log Ratio, p < 0.01).

Lactate, the end product of anaerobic glycolysis, is produced in high amounts by innate immune cells during inflammatory activation, including LPS treatment. However, LPS-induced lactate production in monocytes did not significantly differ between baseline and 6 h postprandially for any of the shakes (Fig. 6e).

### 3.6. Down-regulated inflammatory phenotype of monocytes parallels suppressed mitochondrial activity after the CREAM consumption

Consistent with the above analysis, Ingenuity Pathway Analysis (IPA) predicted reduced secretion of several cytokines by CREAM compared to the VEGE and AMF interventions (Fig. 7a). This differential effect is also demonstrated by the regulatory network of transcriptome regulation (Fig. S2). Colony-stimulating factor 1 (CSF-1/M-CSF), one of the most important cytokines in inflammatory regulation in monocytes, was identified as the highest activated cytokine by VEGE and also the most suppressed cytokine by CREAM (Fig. 7a).

In the prediction of upstream regulators, all transcription factors activated by VEGE were related to oxidative stress. These include TCF7, SRF, SREBF2, and MRTFA (Fig. 7b). In contrast, CREAM suppressed several mitochondrial homeostasis-related transcription factors, including KLF3, GPS2, and NFE2L2. NFE2L2 (NRF2) is an important transcription factor that responds to oxidative stress and was predicted to be one of the most highly suppressed transcription factors. XBP1, a downstream transcription factor of NFE2L2, was suppressed by CREAM as well. Furthermore, BACH1, which is an upstream negative regulator of NFE2L2 and XBP1, was significantly activated by CREAM (Fig. 7c).

### 3.7. Associations between changes in postprandial metabolites and monocyte gene expression are specific to each intervention

To understand to what extent gene regulation in monocytes is correlated to postprandial changes in circulating metabolites, we performed a regularized canonical correlation analysis (rCCA) between postprandial changes in metabolite levels and the postprandial change in expression of the 787 genes that were differentially regulated postprandially between the three shakes. We observed correlations between monocyte postprandial gene expression and circulating metabolites, which showed little

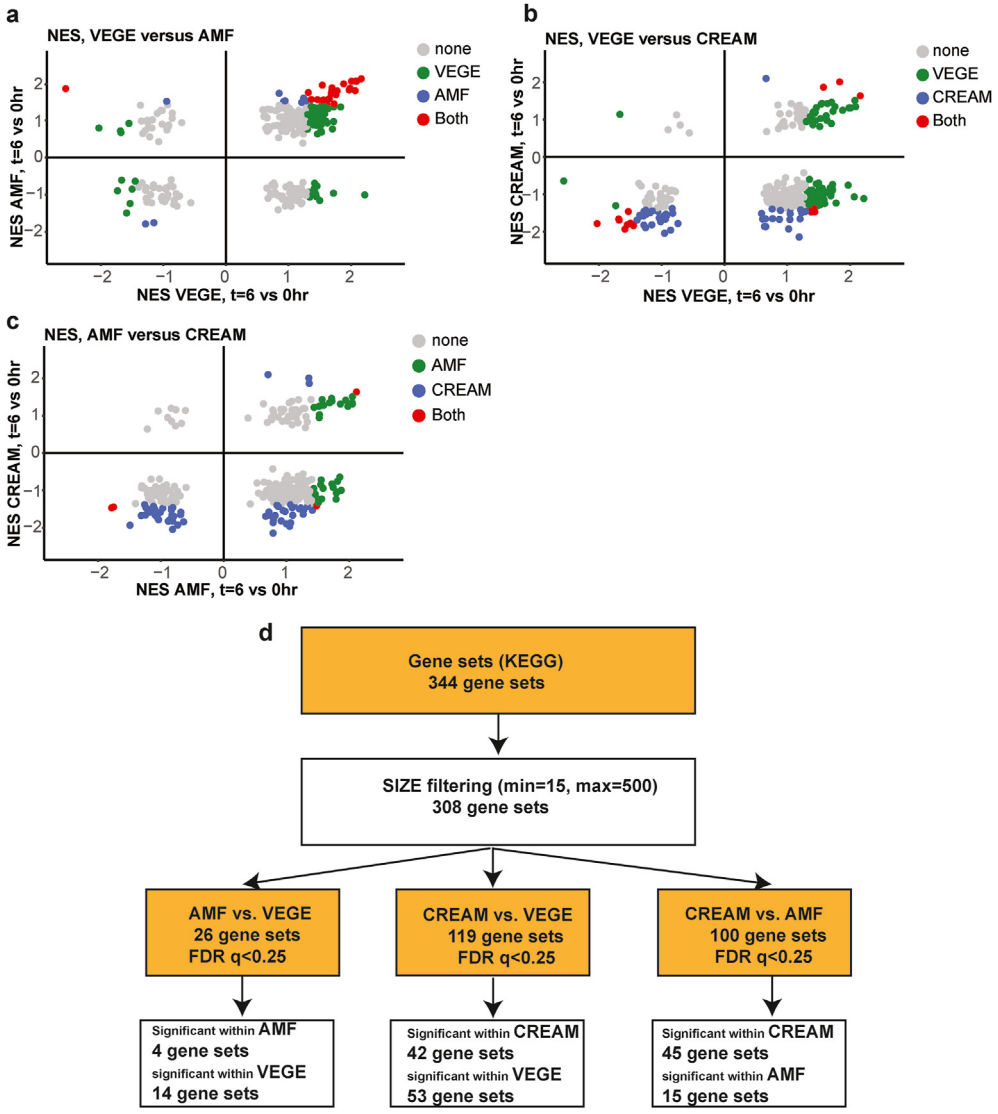


**Fig. 3.** Differential effects of the interventions on KEGG pathways (GSEA analysis). Similarity analysis of regulation on pathways by (a) VEGE and AMF intervention, (b) VEGE and CREAM intervention, and (c) AMF and CREAM intervention. The colored dots represent the significantly regulated pathway. (d) The flowchart shows the number of gene sets differentially changed between interventions (FDR q < 0.25).

consistency between the shakes (Fig. 8a–c). Specifically, we found that the changes in monocyte gene expression after VEGE consumption were correlated to the VLDL profile, LDL profile, fatty acid profile, and phospholipid profile, as well as amino acids, although less pronounced (Fig. 8a). After AMF consumption, a correlation was observed between changes in monocyte gene expression and HDL, VLDL, LDL, and phospholipid profile (Fig. 8b). After CREAM consumption, a correlation was found between changes in

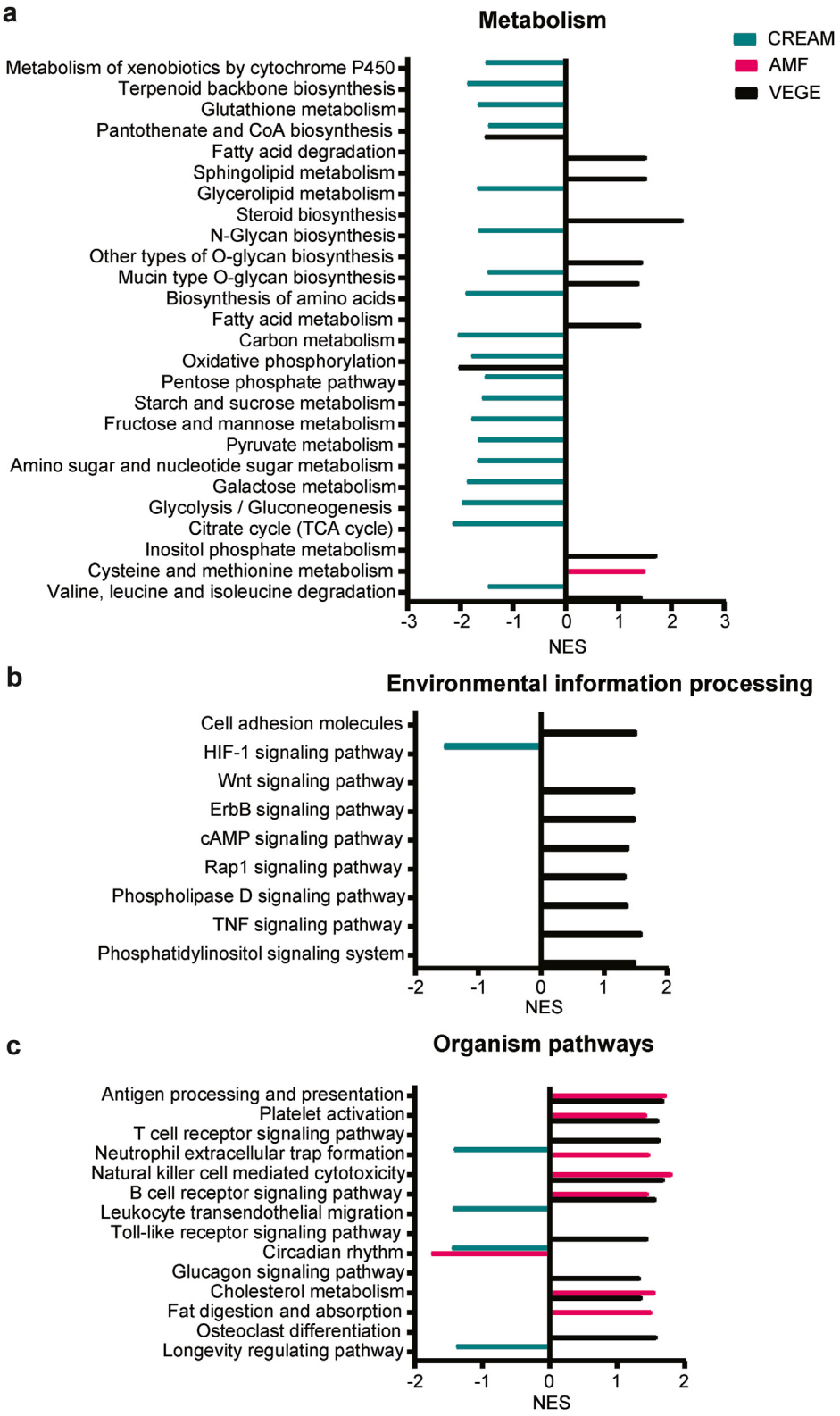


**Fig. 4.** Less activation or inhibitory effects of AMF and CREAM on postprandial metabolism-related pathways (GSEA analysis). Pathways are differentially regulated in between-intervention comparisons and significantly regulated by at least one intervention (FDR q < 0.25) in the KEGG category (a) metabolism, (b) environmental information process, and (c) organism systems by different interventions (FDR q < 0.25).

monocyte gene expression and HDL, VLDL, LDL, and phospholipid profiles. Notably, the genes correlating to the various metabolite profiles differed between shakes.

## 4. Discussion

To gain more insight into the postprandial effect of milk fat and MFGM on monocyte gene expression, a randomized crossover human trial was performed using high-fat shakes made with either a vegetable fat blend, anhydrous milk fat (lacking MFGM), and cream (containing MFGM). We evaluated postprandial changes in monocyte phenotypes and gene expression after consumption of the three shakes and measured ex vivo secretion of TNFα, IL-6, and lactate by these monocytes following LPS stimulation.

The three interventions differentially influenced the monocyte transcriptome, which could not be attributed to changes in the monocyte population. According to flow cytometry, all three shakes caused a significant shift in the monocyte population from a classical phenotype to intermediate and non-classical subsets, yet no differences in postprandial effects were observed between the shakes. Overall, the most distinct effects were observed with CREAM. CREAM consumption was associated with the suppression of energy metabolism pathways, including oxidative phosphorylation, TCA cycle, and glycolysis. VEGE significantly activated fatty acid metabolism-related pathways, while this was not observed with AMF and CREAM consumption. This may be caused by a significantly higher level of postprandial triglycerides in the blood, compared to the other two shakes. One of our major findings is that consumption of VEGE significantly induced inflammation-related pathways, which was observed to a much lesser extent in response to AMF. By contrast, CREAM downregulated several inflammatory pathways, suggesting a potential anti-inflammatory effect of MFGM.

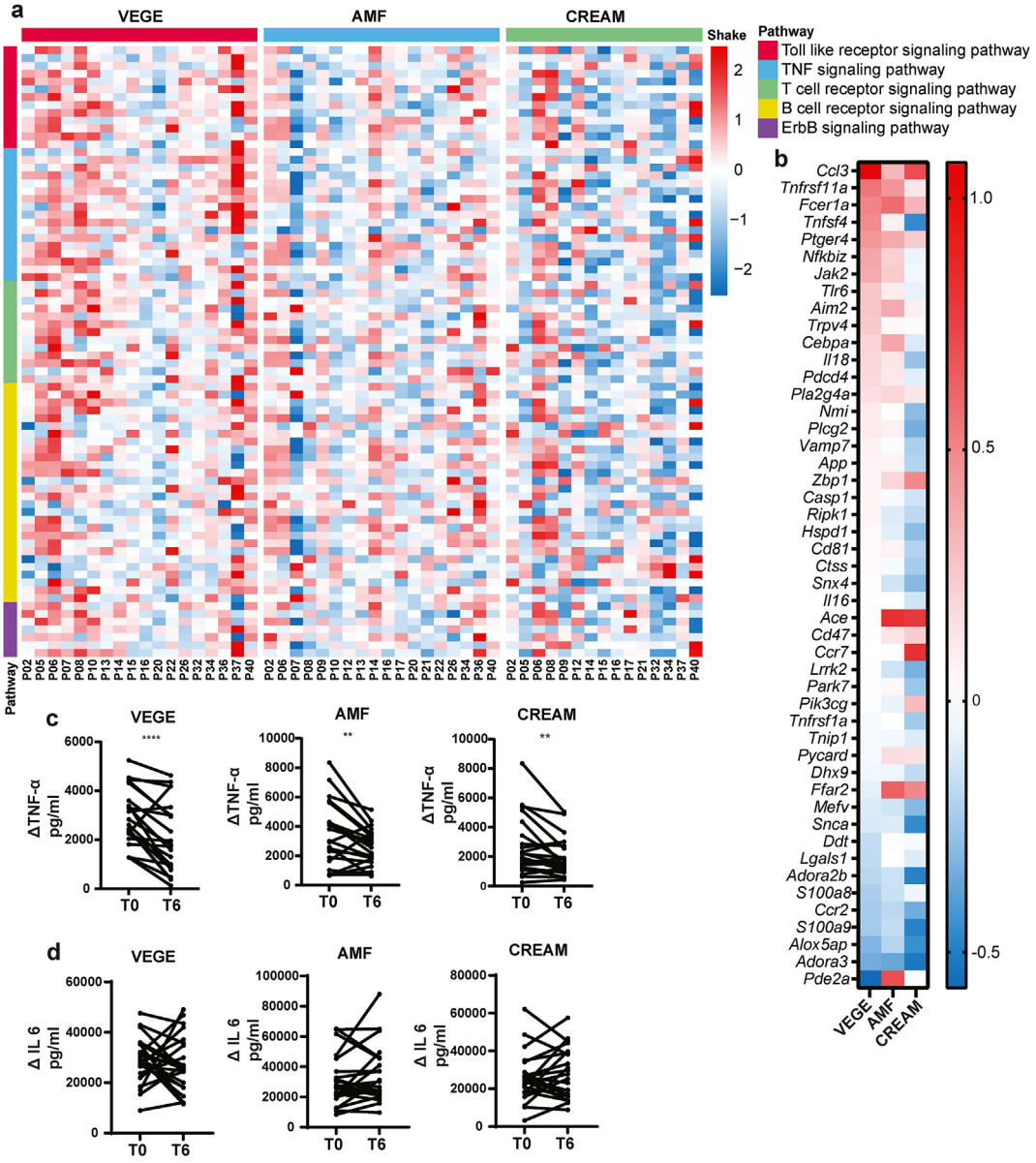


**Fig. 5.** Less activation of inflammatory response-related pathways by AMF and CREAM intervention compared with VEGE intervention. Heatmaps of (a) leading-edge genes in the pathways of interest with differential expression after at least one intervention ($p < 0.05$), (b) genes involved in the inflammatory response and differentially regulated by at least one intervention. (c) The levels of TNFα in the culture medium of monocytes isolated from the participants. (d) IL-6 concentration in the culture medium.

The unique postprandial activation of fatty acid metabolism- and inflammation-related pathways in monocytes by VEGE is potentially linked to the high abundance of PUFA and possibly MUFA in the shake [29–33]. Previously, we studied the postprandial effect of shakes rich in MUFA or SFA on the whole transcriptome in PBMCs [9]. In PBMCs, MUFA increased the expression of inflammatory genes and PPARα target genes involved in fatty acid degradation, which is in line with the observed effect of VEGE in monocytes. In PBMCs, SFA decreased the expression of genes involved in cholesterol biosynthesis and uptake. Despite the high content of SFA in milk fat, we did not observe a significant effect of AMF or CREAM on genes and pathways connected to cholesterol synthesis and uptake as compared to VEGE. These divergent results might be explained by the use of milk fat as a source of SFA in the present study as compared to palm oil in the previous study.

In our study, we found that the phospholipase D signaling pathway, which can be stimulated by PUFA [34–36], was upregulated by VEGE consumption. The phospholipase D pathway regulates intracellular signaling and metabolic pathways. Our data also show activation of cAMP signaling by VEGE, which on the one hand may be a downstream consequence of phospholipase D stimulation and on the other hand may contribute to the inflammatory response [37].

CSF-1 was predicted by IPA to be the most highly induced cytokine by VEGE. The activation of CSF-1 links to the phosphatidylinositol signaling system [38,39] and the cAMP signaling pathway [40,41], which were activated by VEGE in our study. In human monocytes, CSF-1 acts as a key regulator of cell maturation, proliferation, and differentiation [42–44]. Blocking the CSF-1 receptor has been shown to attenuate the secretion of multiple

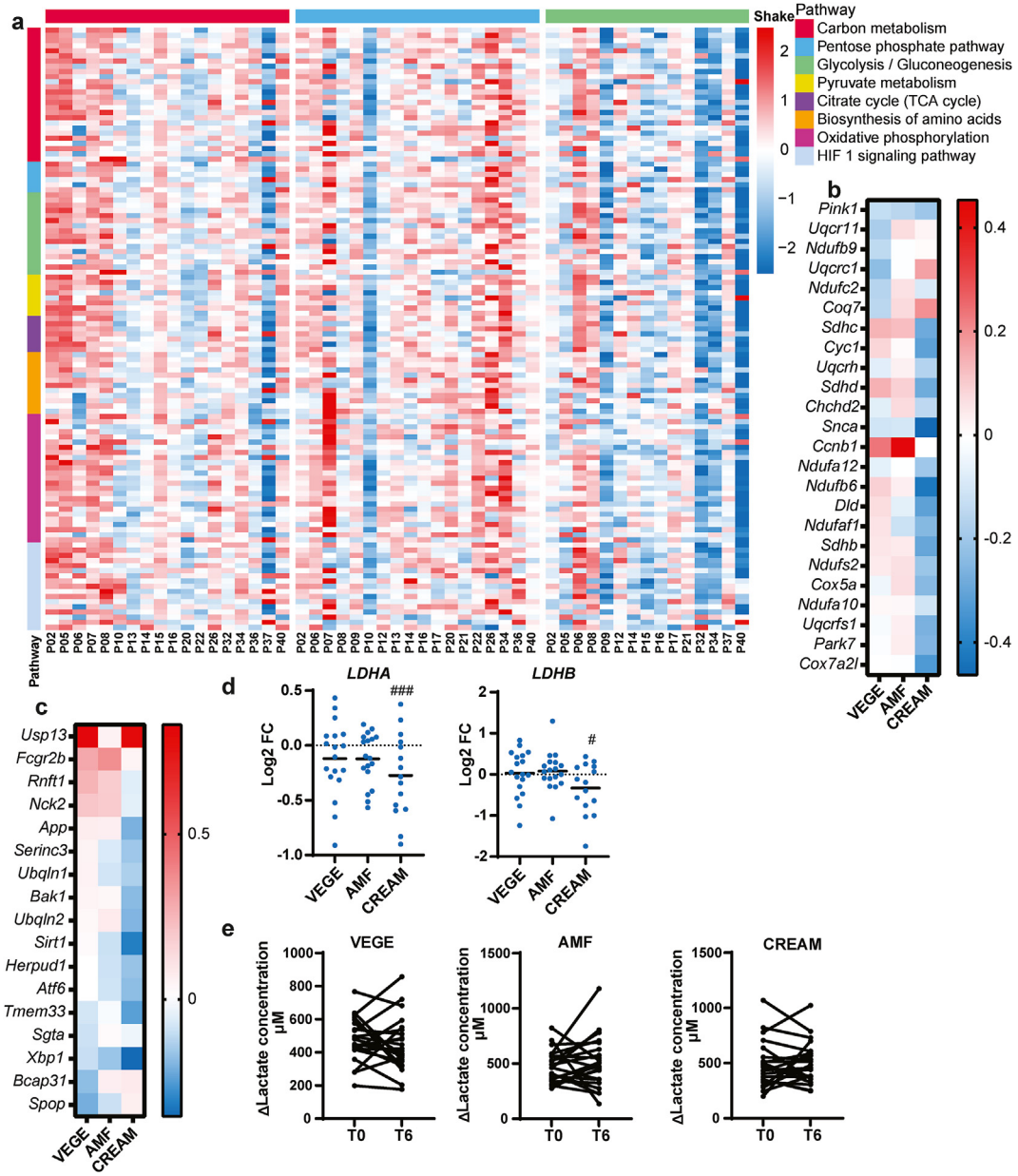


**Fig. 6.** Suppressive effect of the CREAM intervention on energy metabolism-related pathways. Heatmaps of (a) leading-edge genes of pathways of interest in Cytoscape and differentially regulated by at least one intervention, (b) genes involved in ATP electron coupled transportation, and (c) genes involved in oxidative stress. (d) Log 2 fold change of genes related to lactate biosynthesis from pyruvate. (e) The levels of lactate in the culture medium of monocytes isolated from the participants. The isolated monocytes were stimulated with 10 ng/mL LPS overnight.

cytokines by monocytes [45]. Accordingly, it might be that the pro-inflammatory effects of VEGE on human monocytes are potentially mediated by the regulation of CSF-1 expression. It should be noted, though, that we did not observe any difference in the LPS-induced secretion of selected cytokines between the shakes.

CREAM consumption resulted in a reduced expression of genes involved in metabolic processes such as oxidative phosphorylation, TCA cycle, and glycolysis. Since metabolic activation of monocytes is directly coupled to their functional output [46], a reduction in these pathways by CREAM consumption may impact monocyte function. This may partly explain the differential effects on gene expression of inflammatory pathways between the AMF versus the CREAM shake. Since the main difference between AMF and CREAM is the presence of MFGM, we hypothesize that MFGM down-regulates metabolic pathways and further inhibits inflammatory response in monocytes.

Intriguingly, CREAM consumption was predicted to activate the transcription factor BACH1. Genetic BACH1 deficiency has been associated with increased glycolysis and NLRP3 inflammasome activation [47]. In addition, BACH1 was found to modulate the transcription of pyruvate dehydrogenase kinase, thereby decreasing the phosphorylation of pyruvate dehydrogenase in human breast cancer cells [48]. In addition, it has been published that the effect of the transcription factor BACH1 on mitochondrial activity follows the same pattern as HIF-1 signaling [49,50]. Besides hypoxia, HIF-1 is stimulated by immune cell activation and is a key regulator of mitochondrial metabolism and certain immune effector functions [51–56]. Based on these considerations, it is reasonable to hypothesize that the downregulation of energy metabolism by CREAM is potentially linked to the activation of BACH1. Previously, BACH1 was found to function as a hypoxia-inducible repressor for the heme oxygenase-1 gene in many types of human cells [57]. Accordingly, activation of BACH1 may also account for the inhibition of the HIF-1 signaling pathway by CREAM. Although more direct mechanistic evidence is needed, our analysis raises the possibility that MFGM inhibits the HIF-1 signaling pathway by targeting BACH1 with subsequent anti-inflammatory effects at the gene expression level in human monocytes.

Our study also has limitations. First of all, as the study was performed in middle-aged populations, the results can not immediately be translated to other age populations. The principal read-outs of our study were short-term changes in gene expression, which may not translate into changes in protein levels or functional

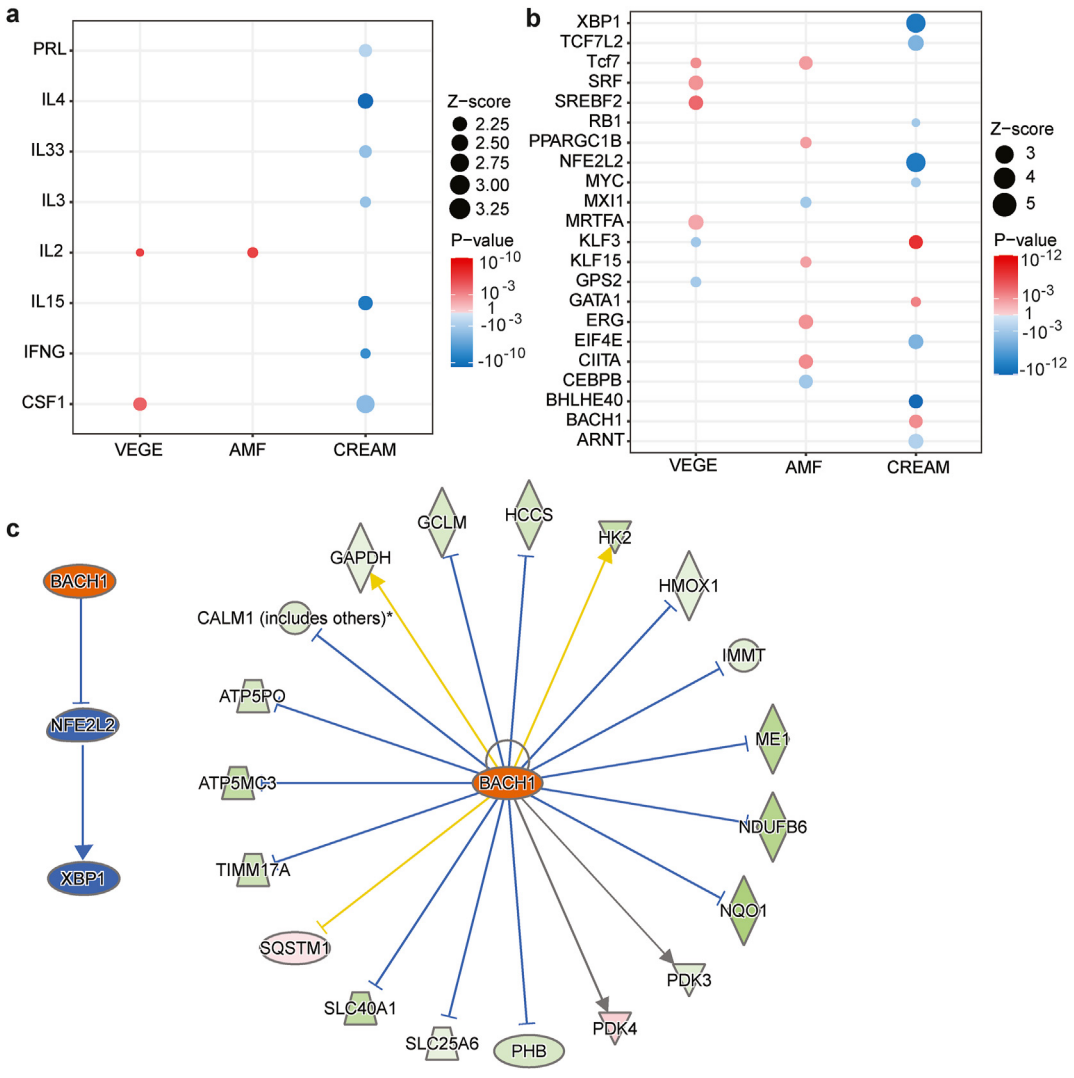


**Fig. 7.** BACH1 is predicted as a master upstream transcription factor in the regulation of mitochondrial metabolism by MFGM. IPA prediction of (a) upstream cytokines and (b) transcription factors regulated by three interventions. (c) Mechanism of prediction of the activation of BACH1 by CREAM intervention and its downstream targets.

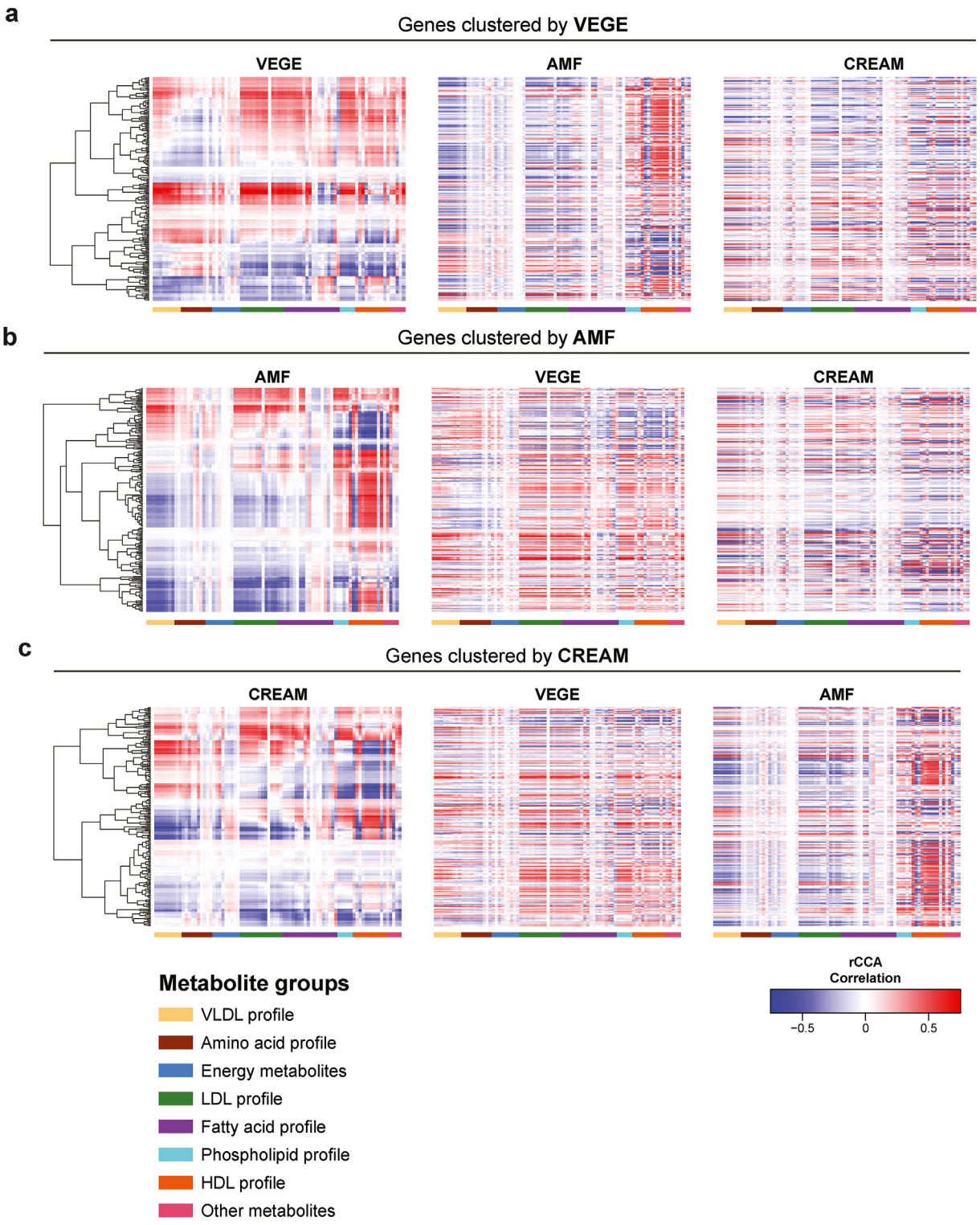


**Fig. 8.** Intervention-specific associations between postprandial metabolites change and regulation of monocyte genes. Correlation analysis between postprandial metabolite changes and monocyte gene regulation in (a) VEGE intervention, (b) AMF intervention and (c) CREAM intervention.

changes. In addition, it is unclear what the long-term consequences are of the observed changes in gene expression. Finally, we used IPA to generate hypotheses about the mechanisms underlying the observed changes in gene expression.

In conclusion, our study has provided detailed insight into the postprandial effect of milk fat on the monocyte transcriptome and has highlighted potential underlying mechanisms. Compared to acute consumption of a vegetable fat blend and anhydrous milk fat, cream significantly downregulated energy and attenuated the activation of proinflammatory pathways in blood monocytes, which were significantly upregulated by the other two interventions, suggesting a potential anti-inflammatory effect of MFGM.

## Author contributions

The study was designed by Lydia A. Afman, Sander Kersten, Anouk L. Feitsma, Rinke Stienstra and Lei Deng. Lei Deng and Charlotte C.J.R Michielsen performed the study with help from Frank Vrieling. Data were processed and interpreted by Lei Deng. Guido J. Hooiveld and Frank Vrieling contributed to RNA-seq data analysis. Lei Deng wrote the first draft. Sander Kersten, Lydia A. Afman, Rinke Stienstra and Frank Vrieling revised the manuscript.

## Data sharing statement

The RNA sequencing data has been made available at the Gene Expression Omnibus (GEO), under accession number GSE249787. Other relevant data are available upon request.

## Conflict of interest

Anouk L. Feitsma is employed by FrieslandCampina. The authors declare that they have no other known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2023.11.038.

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