读书报告

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RESEARCH ARTICLE

A safflower oil based high-fat/high-sucrose diet modulates the gut microbiota and liver phospholipid profiles associated with early glucose intolerance in the absence of tissue inflammation

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Scope: Omega-6 (*n*-6) PUFA-rich diets are generally considered obesogenic in rodents. Here, we examined how long-term intake of a high-fat/high-sucrose (HF/HS) diet based on safflower oil affected metabolism, inflammation, and gut microbiota composition.
Methods and results: We fed male C57BL/6J mice a HF/HS diet based on safflower oil—rich in *n*-6 PUFAs—or a low-fat/low-sucrose diet for 40 wk. Compared to the low-fat/low-sucrose diet, intake of the safflower-based HF/HS diet only led to moderate weight gain, while glucose intolerance developed at week 5 prior to signs of inflammation, but concurrent with increased levels of linoleic acid and arachidonic acid in hepatic phospholipids. Intake of the HF/HS diet resulted in early changes in the gut microbiota, including an increased abundance of *Blautia*, while late changes coincided with altered inflammatory profiles and increased fasting plasma insulin. Analysis of immune cells in visceral fat and liver revealed no differences between diets before week 40, where the number of immune cells decreased in the liver of HF/HS-fed mice.
Conclusion: We suggest that a diet-dependent increase in the *n*-6 to omega-3 (*n*-3) PUFA ratio in hepatic phospholipids together with gut microbiota changes contributed to early development of glucose intolerance without signs of inflammation.

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glucose intolerance / hepatic lipid metabolism / inflammation / leucocytes / safflower oil

Additional supporting information may be found in the online version of this article at the publisher's web-site

主要内容

1 Introduction

1.1 n-6 PUFAs

① **传统观念**：植物油富含omega-6 (n-6) PUFAs，比饱和脂肪更健康。

卫生组织推荐向动物饲料中添加更多的植物油脂

② **迎来挑战**：流行病学研究和很多动物实验表明，过多地摄入n-6 PUFAs 与肥胖症的发生相关。

③ n-6/n-3 PUFAs (Obesogenic effect);

④ **动物试验；高脂日粮诱导；**

饱和油脂（猪油）→ n-6 PUFAs

- 代谢
- 葡萄糖耐受性
- 胰岛素敏感度
- 全身性慢性炎症

1.2 Energy-dense diets，microbiota，host metabolism，information

① 一般来说，高脂高糖饮食会导致全身性慢性炎症，进而引起胰岛素敏感度下降。

② 局部炎症对于白色脂肪组织的扩张是必需的（机体适应性反应）。

③ 通过直接或者间接形成一些免疫调节介质，饮食中某一特定营养物质的高水平添加都可能像高脂饮食一样引发炎症。

④

2 Materials and methods

Supplementary table 3. Macronutrient composition and fatty acid profile of diets.

Diet	HF/HS	LF/LS
Ingredients	%w	%w
Casein	20	20
Corn starch	-	52
Sucrose	43.7	9.7
Cellulose power	5	5
L-Cystine	0.3	0.3
DL-Methionine	0.2	0.2
Vitamin premix	1	1
Mineral & trace element premix	3.5	3.5
Choline chloride	0.3	0.3
Vitamin K3	<0.1	<0.1
Vitamin B12	<0.1	<0.1
Butylated hydroxytoluene	<0.1	<0.1
Dye (Blue / Green)	<0.1	<0.1
Na Carboxymethylcellulose	0.1	0.1
Soybean oil	2.5	7
Safflower oil	22.5	-
Macronutrients	%E	%E
Carbohydrate	42.8	67.6
Fat	42.1	14.3
Protein	13.3	16.0
Fiber	1.8	2.1
Fatty acids	%	%
C14:0	0.2	0.3
C16:0	7.3	12.0
C16:1	0.1	0.1
C18:0	2.7	3.3
C18:1n9	15.3	23.6
C18-1n7	0.5	1.7
C18:2n6	72.9	53.3
C18:3n6	0.4	0.3
C18:3n3	0.7	5.4
SAT	10.2	15.6
MUFA	15.9	25.4
PUFA	73.9	59.0
n-6/n-3 ratio	105	9.9

Legend. Ingredients (per cent of weight) in diet, energy (per cent of total energy) derived from macronutrients, per cent of total lipids. SAT: Saturated fatty acid.

2.1 Animals

Male mice (8 wk of age) (Taconic, Ejby, Denmark)

Low-fat/low-sucrose (**LF/LS**) diet (21–24 ℃) 12-h light and dark cycle

Acclimatization (2 wk)

Half (n = 4 per study, replicated once) were shifted

Safflower oil based high fat/high sucrose (HF/HS) diet **40 wk**

Food intake was recorded twice a week , **body weight** once a week

Body composition was measured by MRI scan

2.2 Indirect calorimetry 间接测热法

After 4 wk on diets and a **3-day** acclimatization period, activity, O_2 and CO_2 gas exchange measurements were determined for a **72-h period**.

2.3 Insulin and glucose tolerance test

Insulin tolerance test: 0.75 mU human insulin per gram lean body mass was injected i.p. after a **2-h fast**.

Glucose tolerance test: mice were injected i.p. with 3 mg glucose/g lean body mass after a **6-h fast**.

Plasma insulin: tail vein/ EDTA tubes.

Blood glucose: glucometer (Contour, Bayer).

2.4 Termination and tissue harvest

Anesthetize the mice before dissection.

Collect venous blood

For RNA,

liver samples: Ambion RNAlater (Invitrogen) overnight at 4℃;

epididymal white adipose tissue (eWAT) samples: flash frozen.

Luminal contents from cecum and colon: flash frozen.

stored at −80 ℃

For for histological analyses,

Liver and eWAT samples: 4% paraformaldehyde.

stored at RT

2.5 Flow cytometry 流式细胞术

Liver and spleen tissue

Supplementary table 1. Antibodies

Panel	Antibodies/conjugates/clones							
Dendritic cell	CD11b V500 (M1/70)*	CX3CR1-Pacific Blue (polyclonal)†	CD45 APC-Cy7 (30-F11)*	*IL-12p35 APC* (4D10p35)†	CD11c PE-Cy7 (HL3)*	Siglec-H PerCP-eFluor- 710 (440c)†	CD103 PE (M290)*	F4/80 FITC (BM8)†
Macrophage	CD11b V500 (M1/70)*	*CD206 BV421 (C068C2)‡*	CD45 APC-Cy7 (30-F11)*	*Arg1 AF647* (19/Arginase-1)*	CD11c PE-Cy7 (HL3)*	Ly6C PerCP-Cy5.5 (HK1.4)†	*TNFa PE* (MP6-XT22)*	F4/80 FITC (BM8)†
Monocyte	CD11b V500 (M1/70)*	CX3CR1 Pacific Blue (polyclonal)†	CD45 APC-Cy7 (30-F11)*	CD115 APC (AFS98)†	Ly6G PE-Cy7 (1A8)†	Ly6C PerCP-Cy5.5 (HK1.4)†	CCR2 PE (polyclonal)§	F4/80 FITC (BM8)†
Granulocyte	Empty	cKit V450 (2B8)*	CD45 APC-Cy7 (30-F11)*	*IL-4 APC* (11B11)†	Ly6G PE-Cy7 (1A8)†	FcERIa PerCP-eFluor- 710 (MAR-1)†	Siglec-F PE (E50-2440)*	CD49b FITC (HMa)†
B cell	B220 V500 (RA3-6B2)*	IgD eFluor 450 (11-26c)†	CD45 APC-Cy7 (30-F11)*	*IL-10 APC* (JES5-16E3)†	IgM PE-Cy7 (II/41)†	CD19 PerCP-Cy5.5 (1D3)†	CD1d PE (1B1)†	CD5 FITC (53-7.3)†
T cell	CD3 V500 (500A2)*	TCRgd BV421 (GL3)†	CD45 APC-Cy7 (30-F11)*	*FoxP3 AF647* (FJK-16s)*	CD4 PE-Cy7 (GK1.5)†	*IFNg PerCP-Cy5.5* (XMG1.2)*	*IL-4 PE* (11B11)†	CD8 FITC (53-6.7)*
NK/NKT	CD3 V500 (500A2)*	TCRgd BV421 (GL3)†	CD45 APC-Cy7 (30-F11)*	*IL-4 APC* (11B11)†	NKp46 PE-Cy7 (29A1.4)†	*IFNg PerCP-Cy5.5* (XMG1.2)*	*IL-17A PE* (17B7)†	NK1.1 FITC (PK136)*

Rows refer to panels, Columns to antibodies in panel. Clone marked by parenthesis. *BD Biosciences, †eBioscience, ‡Biolegend, §R&D Systems.

树突细胞

巨噬细胞

单核白细胞

粒性白细胞

B细胞

T细胞

自然杀伤细胞

2.6 Reverse transcription and qPCR

Trizol (Ambion) → **RNA**

Nanodrop 2000 Spectrophotometer

1 μg liver and 100 ng eWAT RNA → **cDNA**

RevertAid Reverse Transcriptase (Thermo Scientific, USA)

SensiFAST SYBR Lo-ROX kit (Bioline, USA)

Mx3000P system (Agilent Technologies, USA) → **RT-PCR**

The thermal profile:
95℃, 5min;
95 ℃, 15 s,
55–63 ℃, 20 s,
72 ℃, 15 s.
} 40 cycles

Supplementary table 2. Primers used.

Gene	Forward primer, 5'-	Reverse primer, 5'-
G6pc	ACCGGACCAGGAAGTCCC	GCAATGCCTGACAAGACTCC
Pepck	GTGCCTGTGGGAAGACTAAC	CCTTAAGTTGCCTTGGGCAT
Cpt1a	TACTGCTGTATCGTCGCACG	GACGAATAGGTTTGAGTTCCTCAC
Acaca	TGCTGCCCCATCCCCGGG	TCGAACTCTCACTGACACG
Ppara	AGAGAGGACAGATGGGGCTC	CGTTTGTGGCTGGTCAAGTT
Srebp1	GGAGCCATGGATTGCACATT	GCTTCCAGAGAGGAGGCCAG
Tnf	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Fasn	ATTGGTGGTGTGGACATGGTC	CCCAGCCTTCCATCTCCTG
Actb	ATGGGTCAGAAGGACTCCTACG	AGTGGTACGACCAGAGGCATAC
Pparg1	GTGTGACAGACAAGATTTGAAAG	GCTTGATGTCAAAGGAATGCG
Pparg2	ACAGCAAATCTCTGTTTTATGC	TGCTGGAGAAATCAACTGTGG
Gapdh	CAAATTCAACGGCACAGTCAA	GTCTCGCTCCTGGAAGATGG
Tbp	ACCCTTCACCAATGACTCCTATG	ATGATGACTGCAGCAAATCGC
Adipoq	GATGGCAGAGATGGCACTCC	CTTGCCAGTGCTGCCGTCAT
Scd	ACACCTGCCTCTTCGGGATT	TGATGCCCAGAGCGCTG

2.7 Extraction and quantification of liver lipids

Samples were weighed and chloroform:methanol (2:1) containing 100 μg/mL butylated hydroxytoluene and internal standards (pentadecanoic acid, di C15:0 phophatidylcholine, and tri C17:0 trialcylglycerol) were added. Samples were homogenized and 20% volume of 0.73% NaCl was added followed by centrifugation (4000 rpm, 5 min, 5°C). The lower phase was retrieved and stored at −20°C. Lipid extracts were fractionated on an aminopropyl cartridge (Phenomenex Strata NH2, 500 mg) as previously described [30].

氯仿:甲醇 (2:1)

100 μg / mL 丁羟甲苯

内标（十五烷酸、磷脂酰胆碱、TG）

0.73% NaCl

固相萃取柱 (Phenomenex Strata NH2, 500 mg)

2.8 Histological examinations

Paraffin embedded sections

Hematoxylin and eosin staining

200 intact cells, three to four sections

ImageJ software.

Adipocyte numbers

Sackmann-Sala, L., Berryman, D. E., Munn, R. D., Lubbers, E. R. et al., Heterogeneity among white adipose tissue depots in male C57BL/6J mice. Obesity 2012, 20, 101–111.

2.9 Bacterial 16S rDNA amplicon sequencing and bioinformatics

Cecal and fecal samples

Greengenes database (4feb2011) **QIIME v1.8.0** Quantitative Insights Into Microbial Ecology

Data filtering

Operational taxonomic units:

a) Present in fewer than three samples;

b) A relative abundance across all samples ≤ 0.005%

Samples containing ≤ 10 000 or ≥ 60 000 sequences

Differences in bacterial composition and abundance

ANOSIM function **Analysis of similarities** 样品组间差异显著性检测

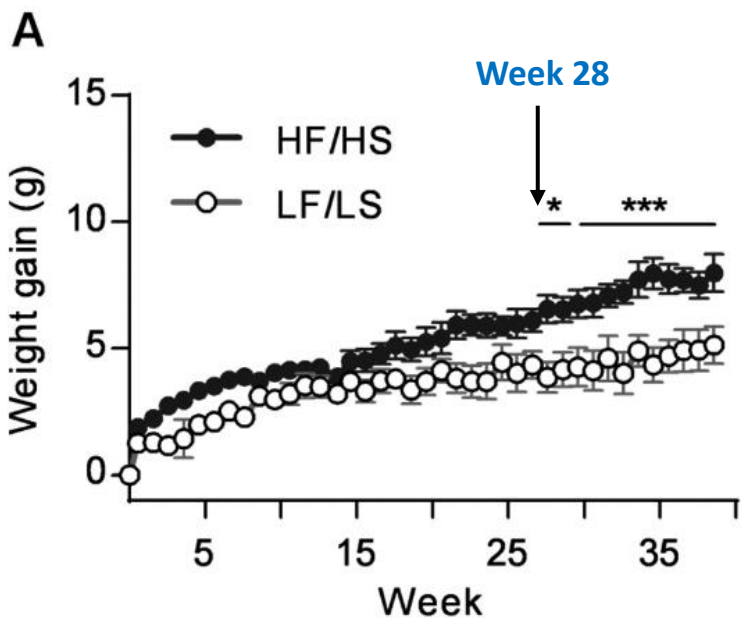
LefSe **Biomarker**

2.10 Statistics

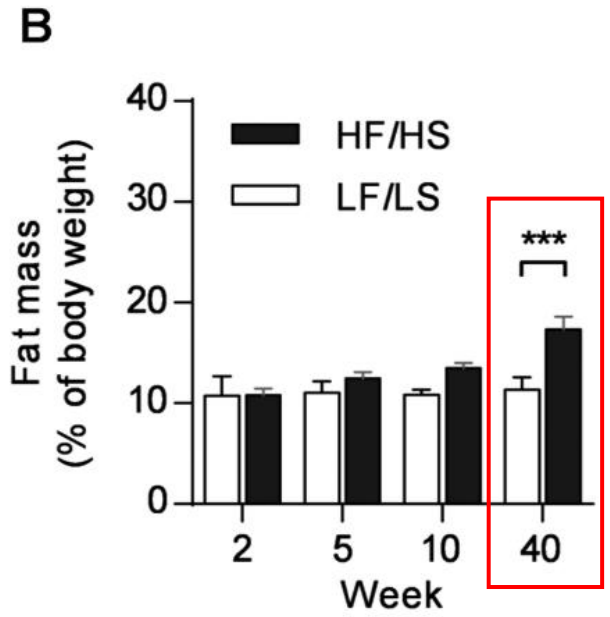
Unless specified, two-way ANOVA (on normally distributed data) was used in GraphPad Prism 6.07. p values lower than 0.05 were considered significant after correction for multiple comparisons by false-discovery rate. Differences between experimental replications were tested using Wilcoxon Rank Sum test as were differences in groups in case $n < 6$ for all time points. Data are presented as mean ± SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Outliers were detected using the Grubbs' test in R v3.1.3.

3 Results

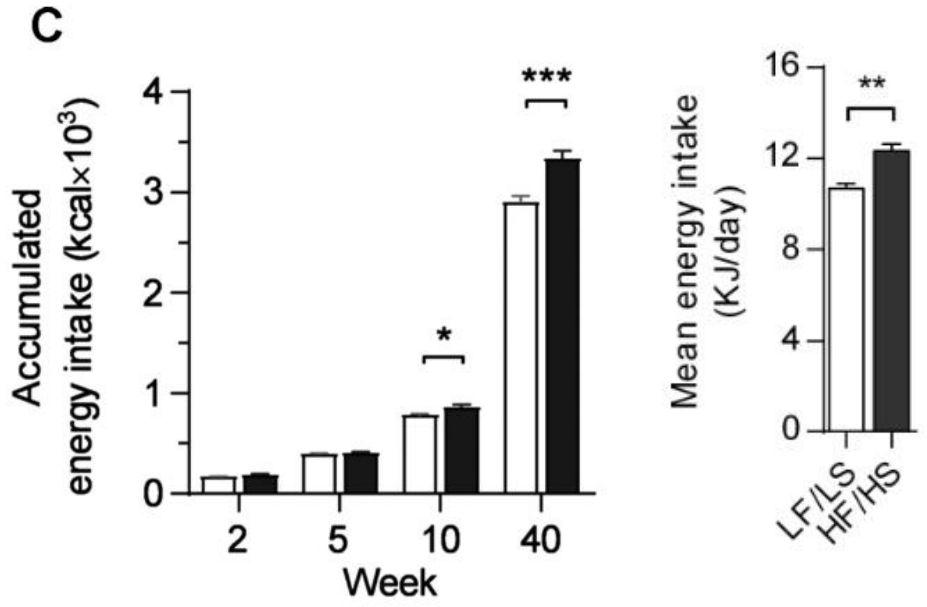
3.1 C57BL/6J mice fed a safflower oil based n-6 PUFA-rich HF/HS diet develop early glucose intolerance in absence of insulin resistance



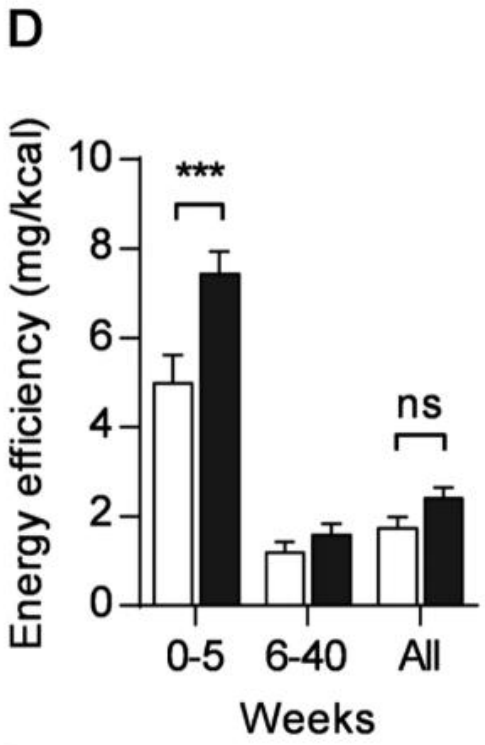
体增重



体脂肪含量占比

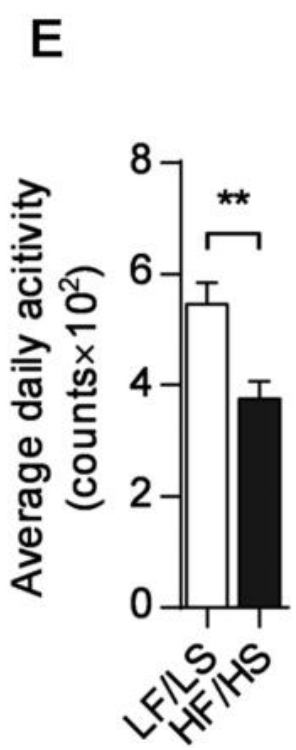


累计能量摄入

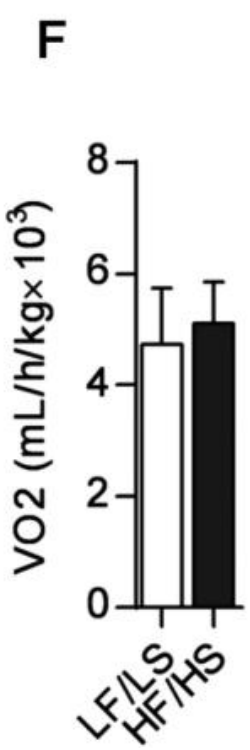


能量转换效率

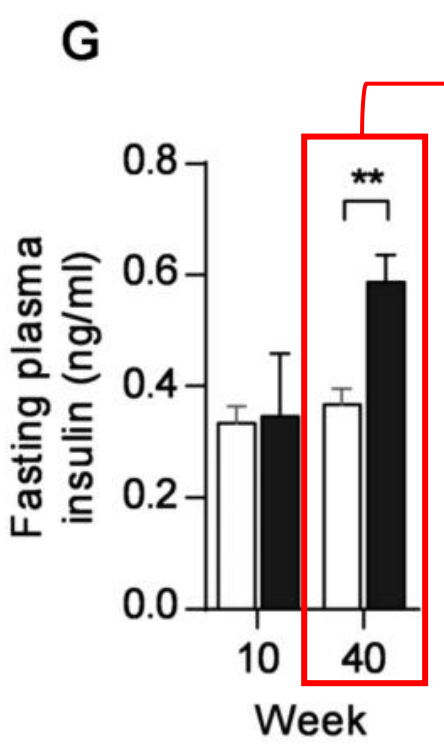
(weight gain relative to energy intake in kcal).



平均日活动量

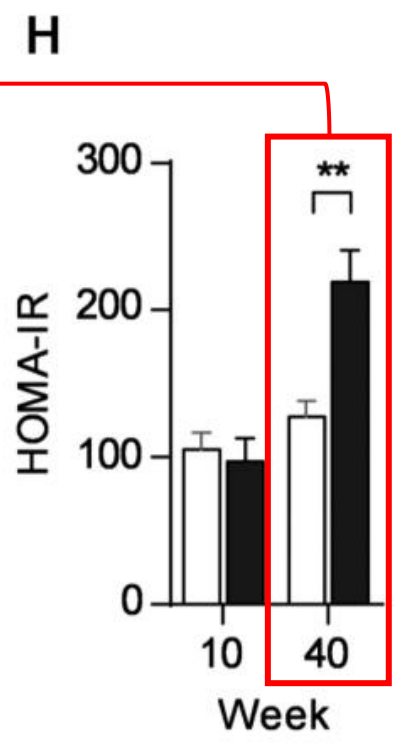


平均日耗氧量



血浆胰岛素水平

（禁食6h）

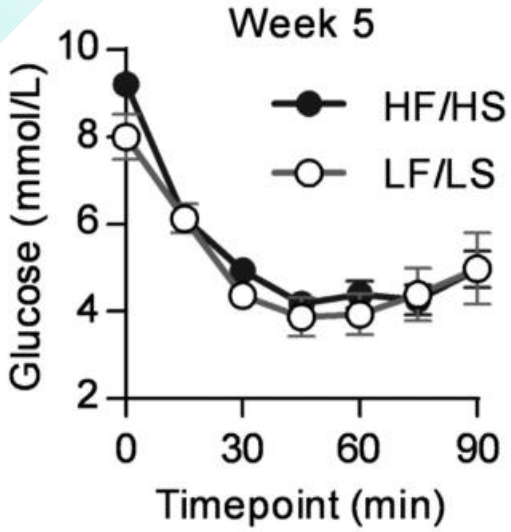


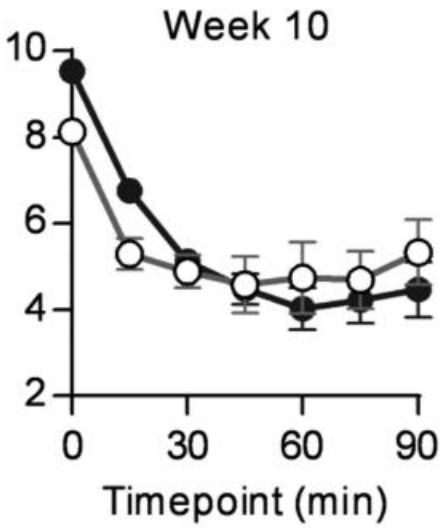
胰岛素抵抗指数

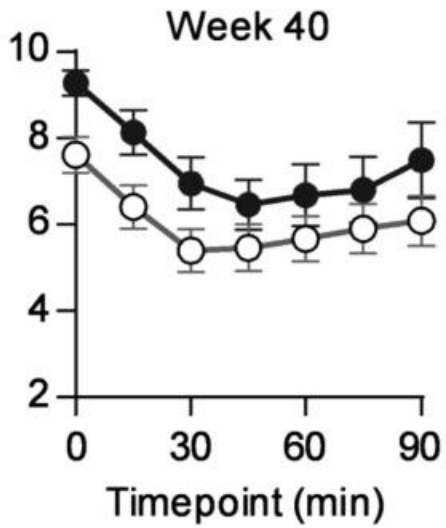
→ Insulin sensitivity

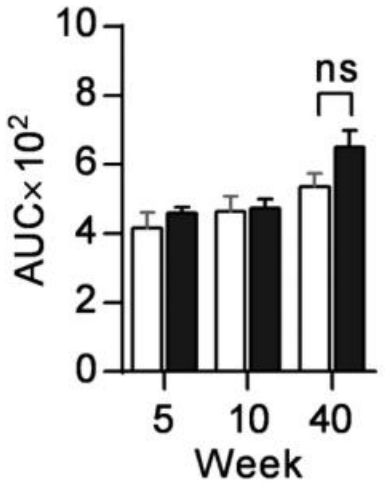
胰岛素耐量试验

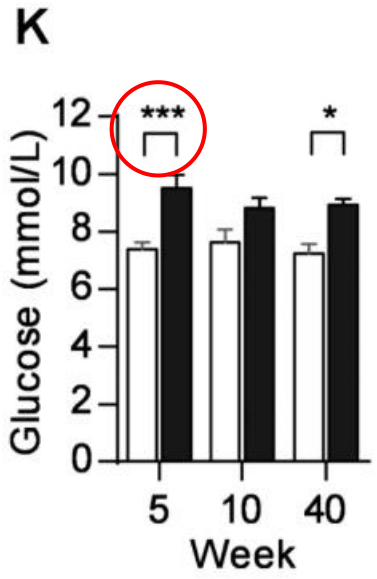
Area under the curve from insulin tolerance test.



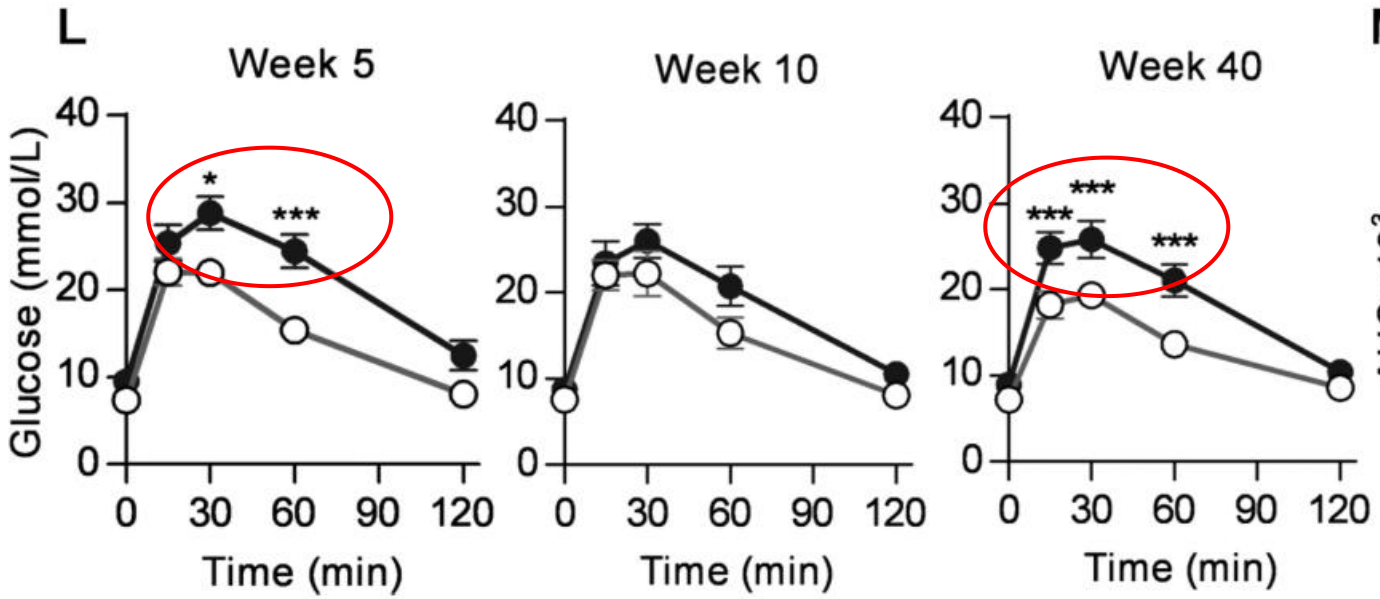


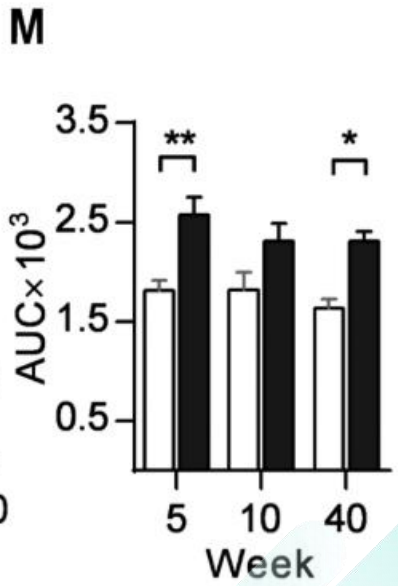






空腹血糖

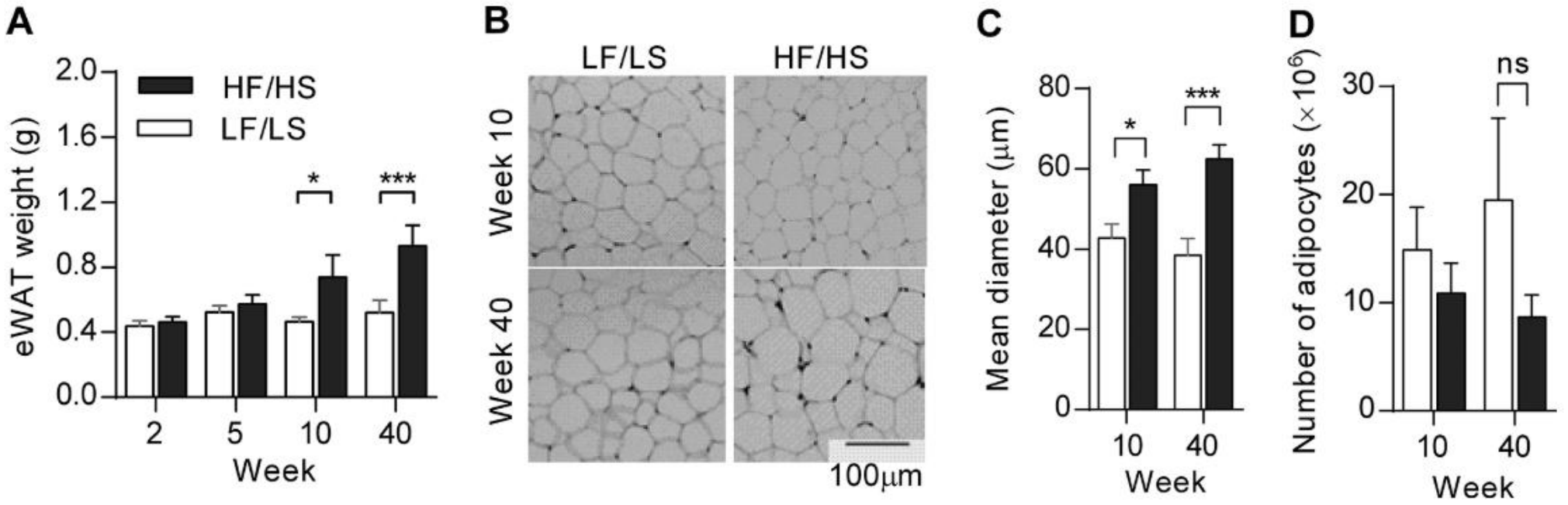


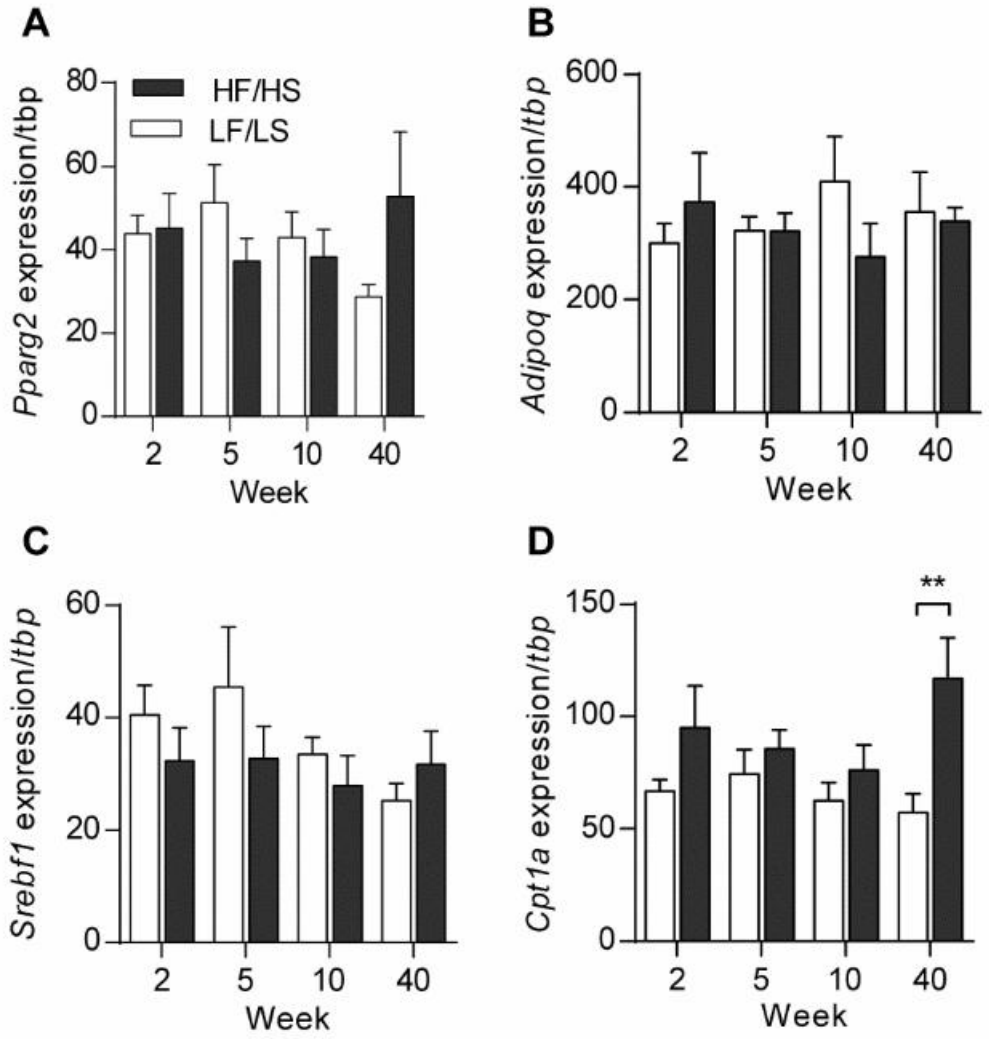


葡萄糖耐量试验

3.2 Intake of a safflower oil based n-6PUFA-rich HF/HS diet results in adipocyte hypertrophy but not in hyperplasia

White squares, LF/LS-fed mice; black squares, HF/HS-fed mice.



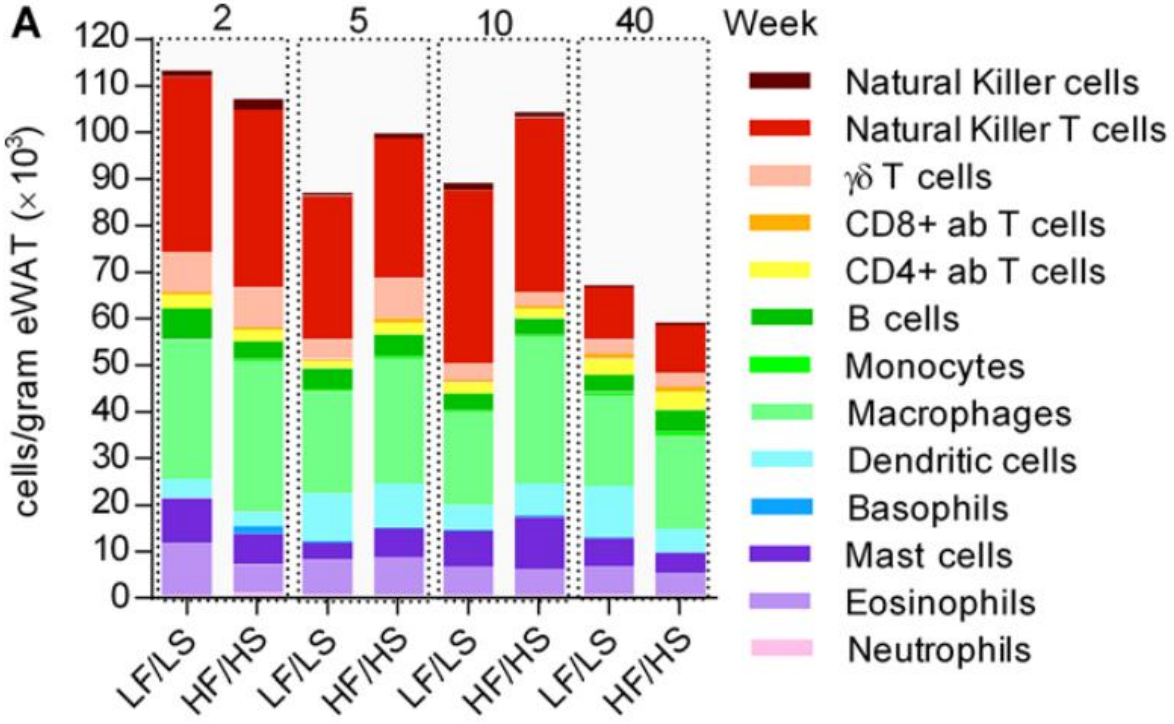


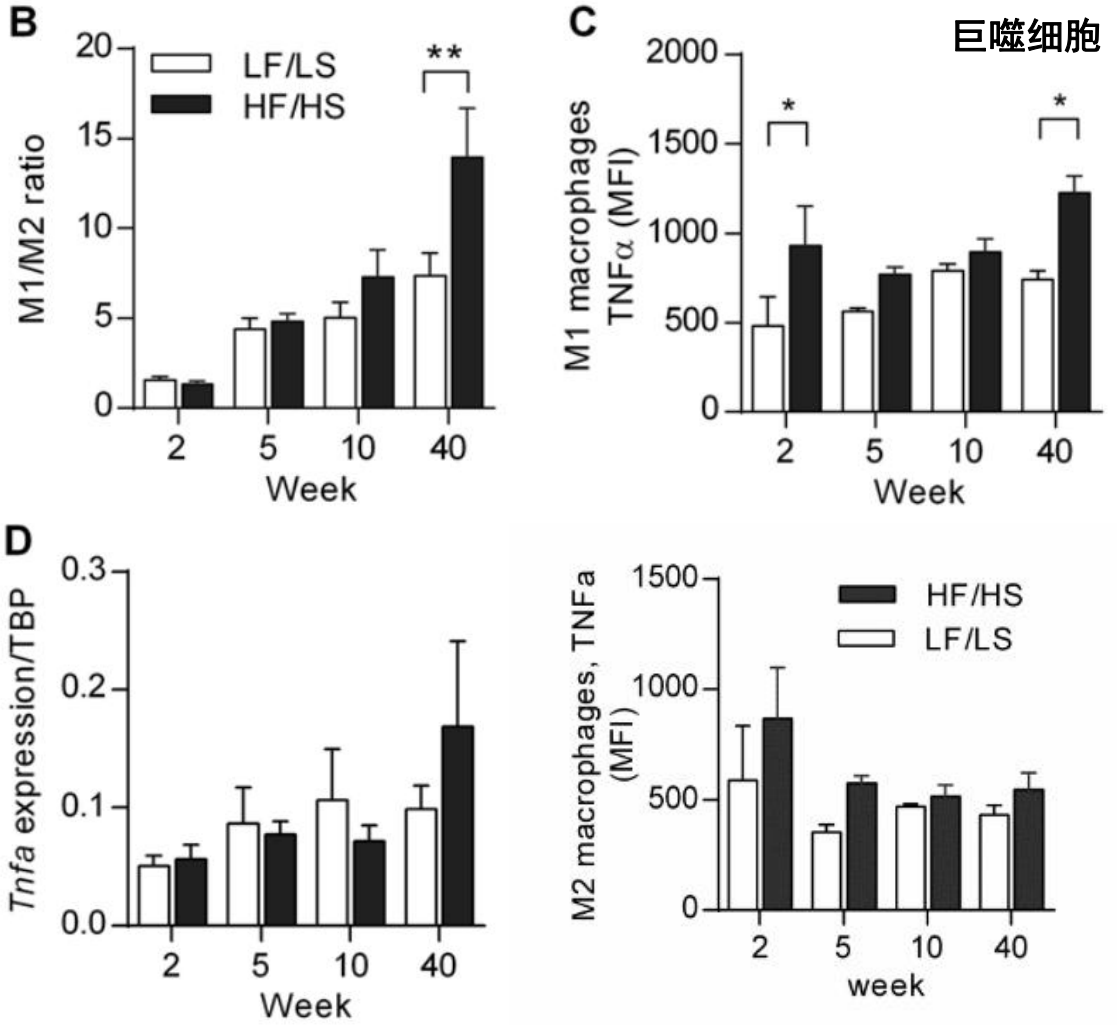
Author:

Thus, the lack of **adipocyte hyperplasia** in response to n-6 PUFA rich safflower oil based HF/HS diets might contribute to the development of persistent glucose intolerance.

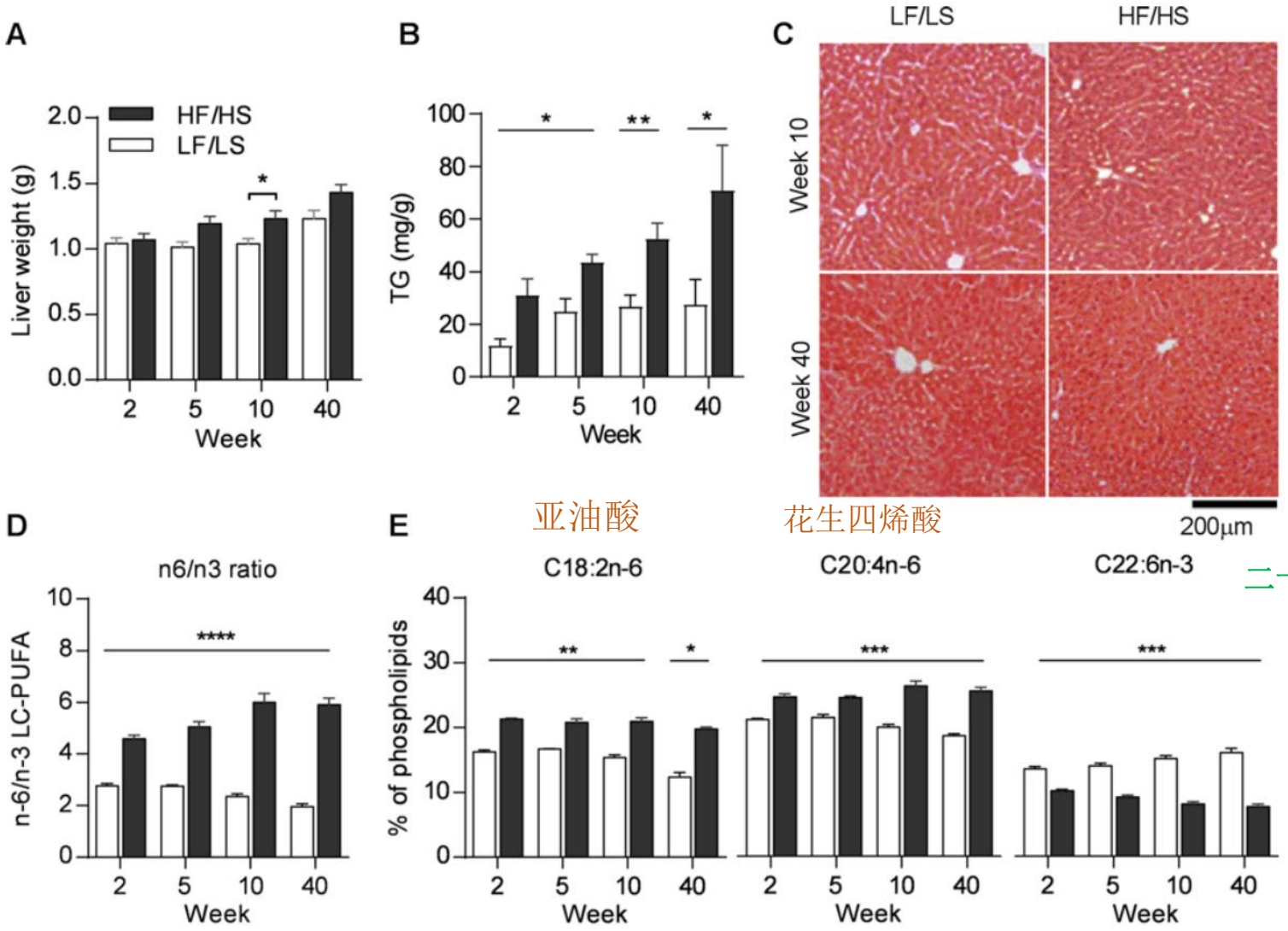
Supplementary figure 3. Expression of genes involved in adipocyte function and lipid metabolism in eWAT. (A) *Pparg2*, **(B).** *Adipoq*, **(C)** *Srebf1*, **(D)** *Cpt1a*. For all data, n=6-8 from two experiments. Black squares = HF/HS-fed mice, white squares = LF/LS-fed mice.

3.3 Glucose intolerance associated with intake of a safflower-oil-based n-6 PUFA-rich HF/HS diet is not dependent on inflammation in eWAT



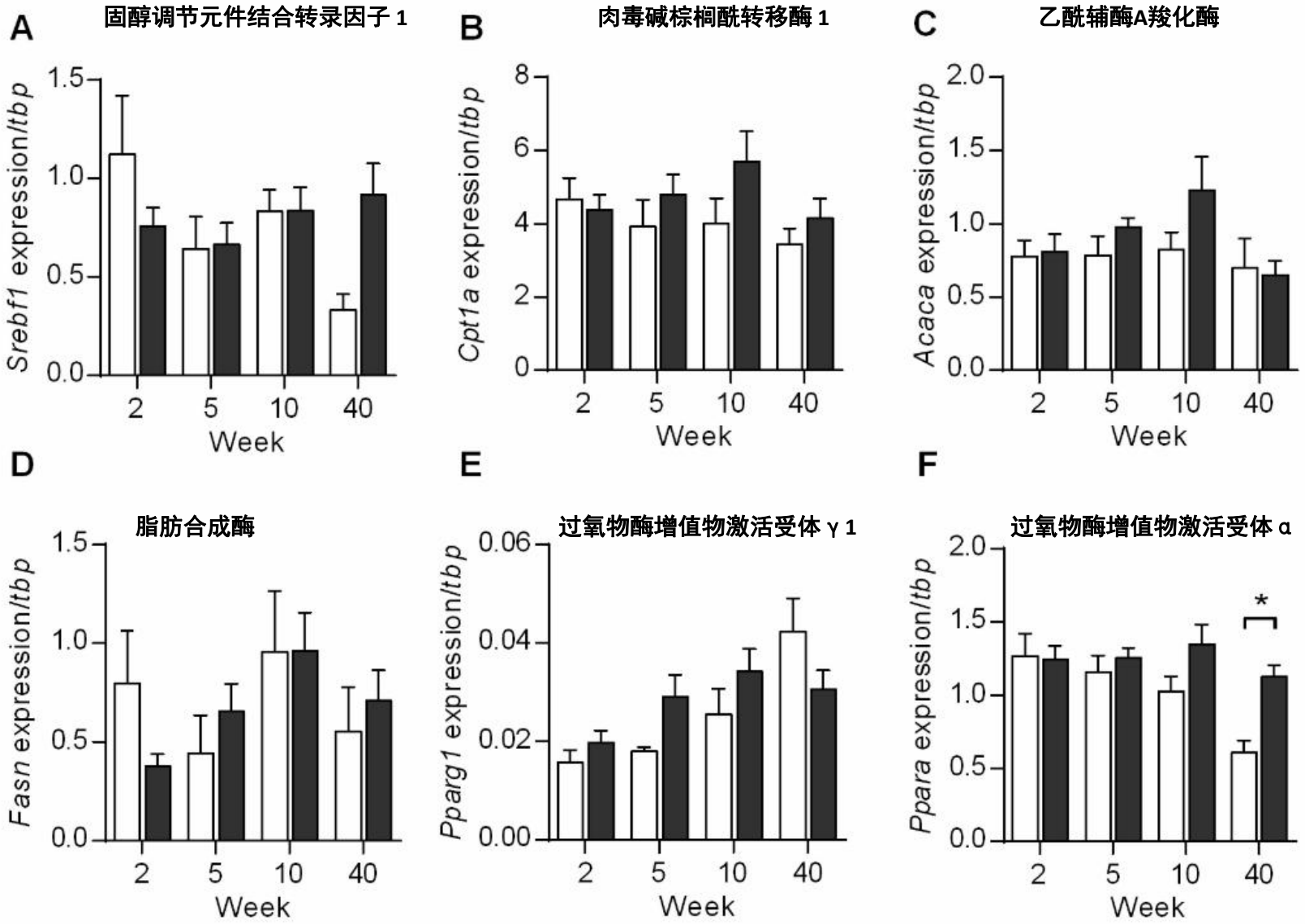


3.4 Intake of a safflower-oil-based n-6PUFA-rich HF/HS diet leads to early changes in hepatic phospholipid profiles without immune cell infiltration and hepatic steatosis

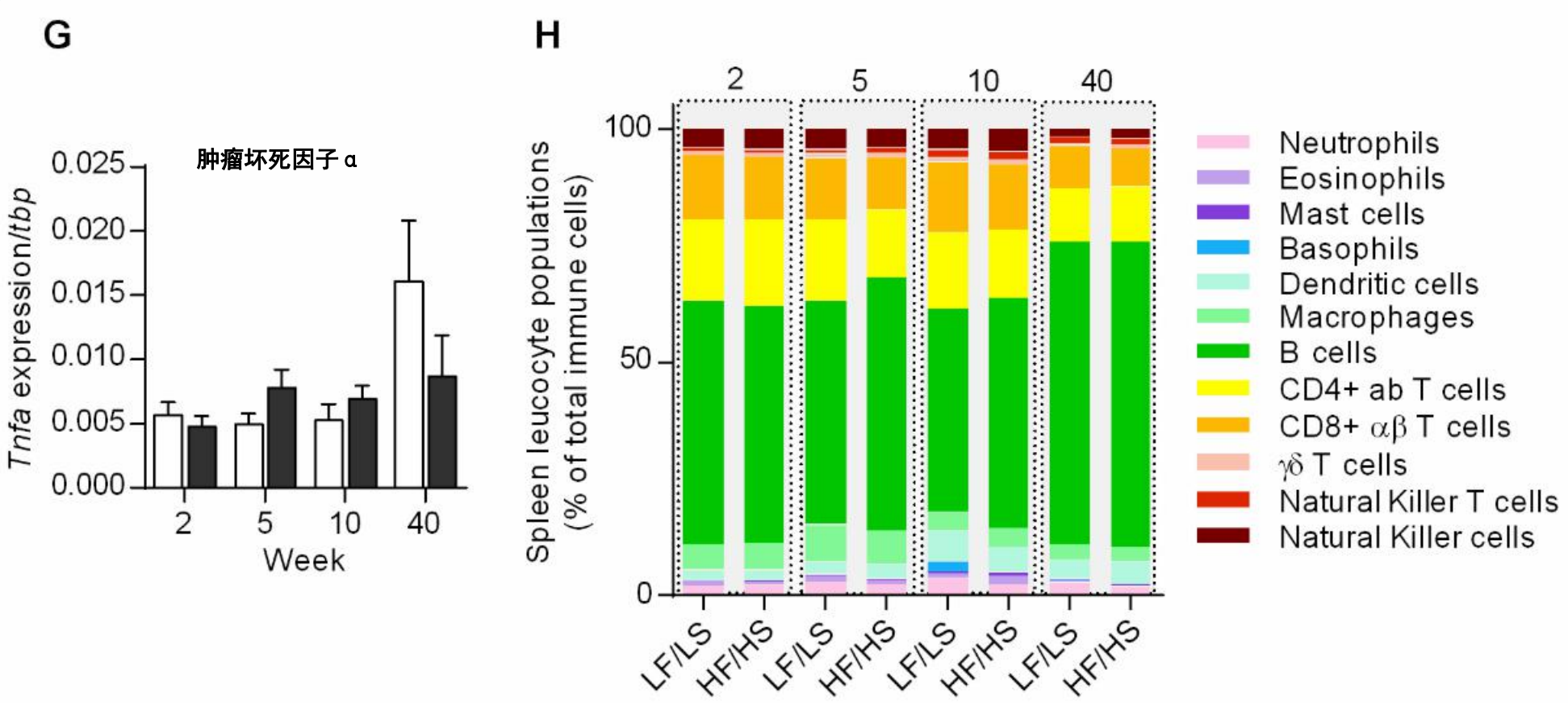


亚油酸 花生四烯酸 二十二碳六烯酸

n-6 PUFAs are important precursors of pro-inflammatory lipid mediators

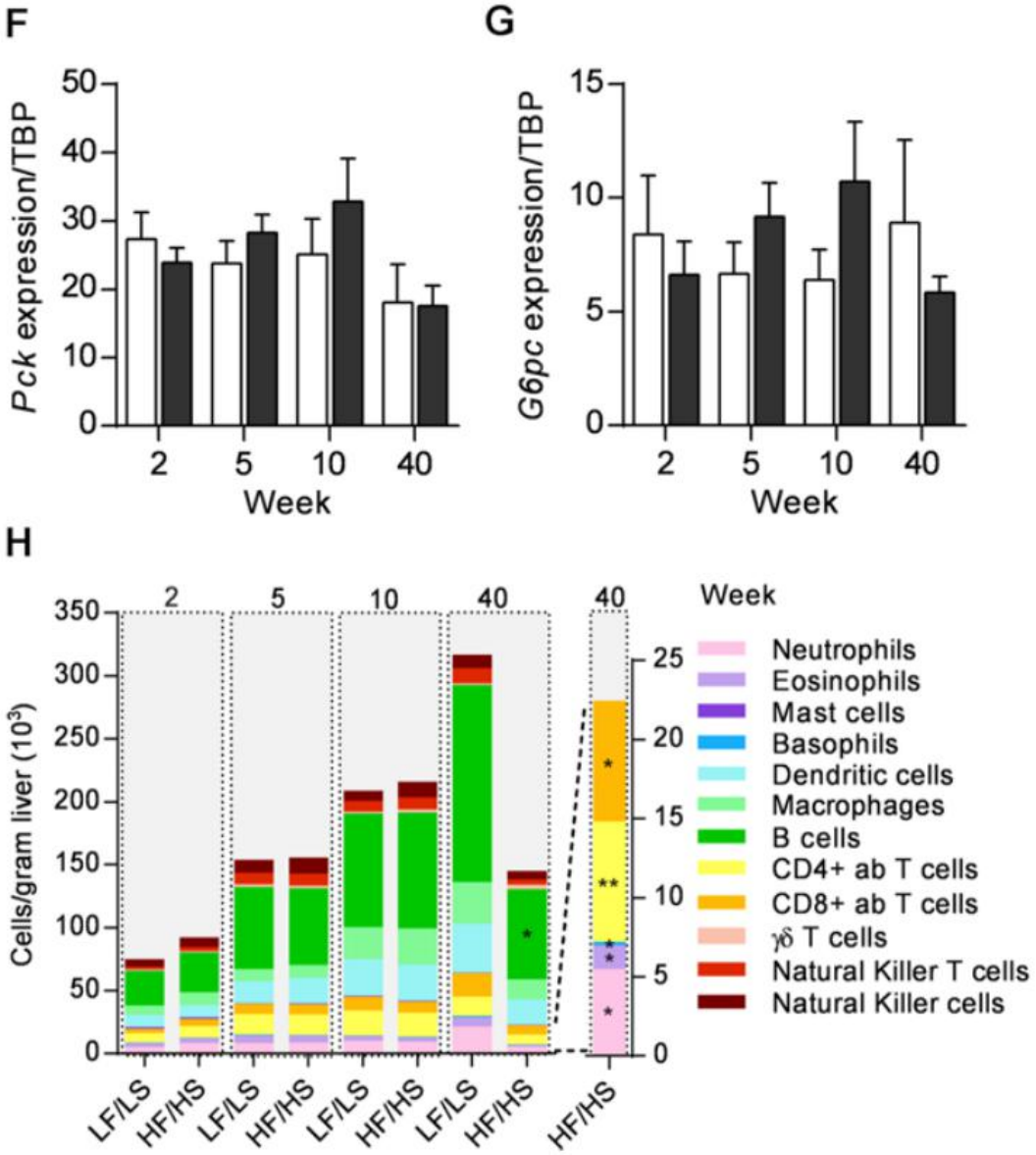


Supplementary figure 5. Hepatic gene expression



Supplementary figure 5. Hepatic gene expression and immune cells in the spleen. (A-G) Hepatic gene expression **(A)** *Srebf1*, **(B),** *Cpt1a*, **(C)** *Acaca*, **(D)** *Fasn*, **(E)** *Pparg1*, **(F)** *Ppara*, **(G)** *Tnfa*. **(H)** Immune cells in the spleen in per cent of total leucocytes. For all data, n=6-8 from two experiments. Black squares = HF/HS-fed mice, white squares = LF/LS-fed mice.

Glucose intolerance.



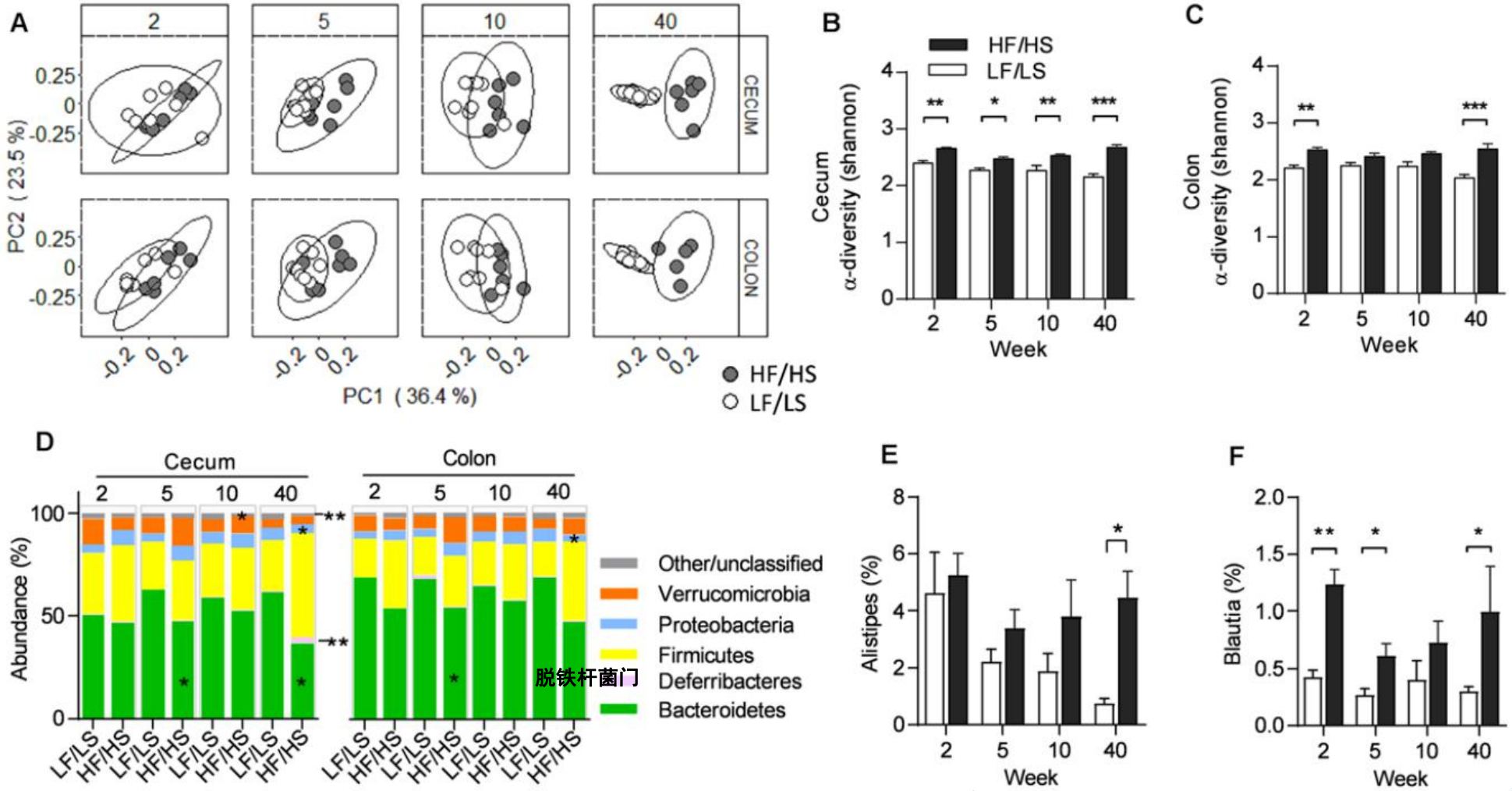
肝脏糖异生相关基因

pck、*G6pc*

Hepatic infiltrating immune cells

Neutrophils
Eosinophils
Basophils
B cells
CD4+/CD8+ ab T cells

3.3 Intake of safflower oil rich HF/HS diet leads to rapid changes in gut bacterial composition



Bacteroides

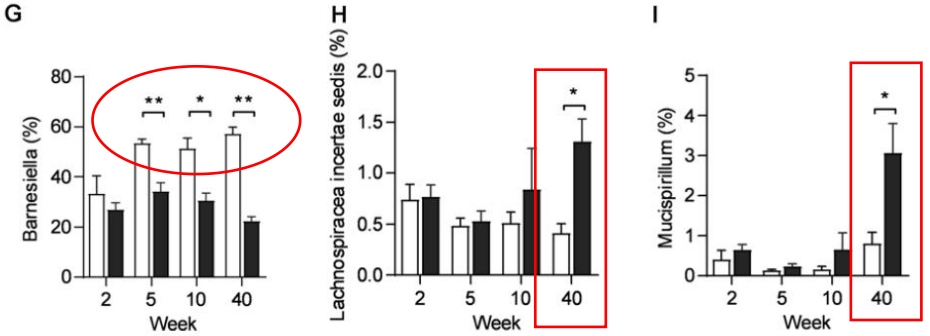
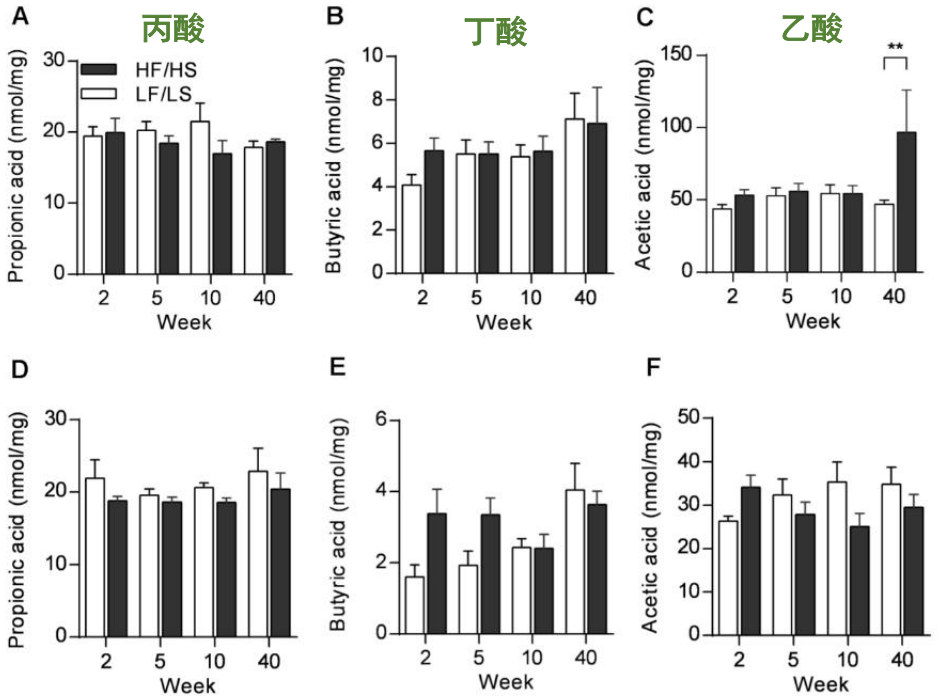
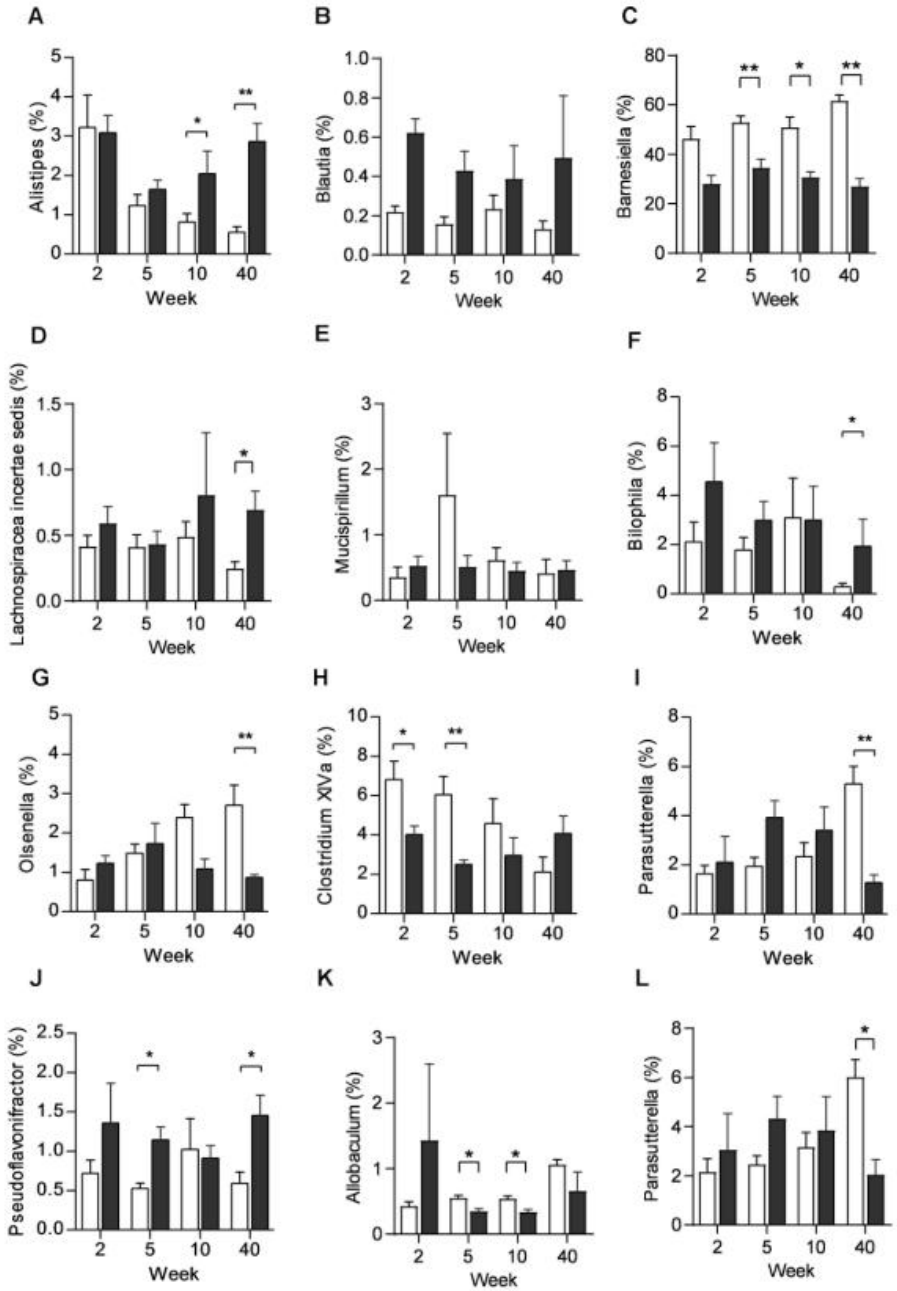


Figure 5. Cecum and colon microbiota composition. (A) Principal coordinates analysis of operational-taxonomic units from 16S rDNA amplicon sequencing of cecal and colonic bacteria. (B) α-diversity in the cecum, and (C) colon. (D) Phylum abundance in the cecum and the colon. (E–I) Changes in relative abundances of genera in the colon (E) and cecum (F–I) with at least one significant difference between diets, a mean abundance above 1% for a diet at any week, and with a consistently lower or higher mean abundance between the diets across weeks. For all data, $n = 6–8$ from two experiments. White circles and squares, LF/LS-fed mice; gray circles and black squares, HF/HS-fed mice.



Supplementary figure 8. SCFAs in cecum and colon. Concentrations of **(A)** propionic acid, **(B)** butyric acid and, **(C)** acetic acid in the cecum. Concentrations of **(D)** propionic acid, **(E)** butyric acid and, **(F)** acetic acid in the colon. For all data, n=6-8 from two experiments. Black squares = HF/HS-fed mice, white squares = LF/LS-fed mice.



Supplementary figure 7. Microbiota composition in cecum and colon of abundant genera with at least one significant difference between HF/HS and LF/LS diets but without difference in abundance. Legend continues on next page.

4 Discussion

1. n-6 PUFA-rich HF/HS diet exhibited early onset of **glucose intolerance** prior to increased **fat mass** and before development of **insulin resistance**.

2. The study design did not allow for correlations between **microbiota** **and** **metabolic** parameters.

3. Changes in **hepatic** **phospholipids** (dominance of the n-6 PUFAs, linoleic acid, and arachidonic acid, at the expense of the n-3 PUFAs).

4. Lower abundance of ***Barnesiella***, Higher abundance of ***Alistipes***.

5. The disparate results may relate to differences in baseline microbiota composition resulting in different responses to high-fat feeding.

6. High levels of safflower oil and sucrose → **highly obesogenic**

思考与感悟：

1. 文章语言地道简练，内容很有嚼头；
2. 设计思路具有前瞻性；
3. 内容丰富，涉及面广；
4. 食物成分、宿主代谢、免疫、微生物种群、代谢产物变化、组织特异性等，构成了一个全面的网络，分析并不简单，仍需大量研究证实；
5. 为自己养殖实验的开展、数据的分析、文章的撰写等方面提供了一定参考。

THANKS