The gut microbiota in cardiovascular disease
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CHAPTER 5

The gut anti-inflammatory agent
5-ASA does not protect against insulin resistance in mice

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Abstract

Objective
5-aminosalicylic acid (5-ASA) is widely used in the treatment of inflammatory bowel diseases (IBD) and was recently shown to improve insulin resistance (IR) in diet-induced obese (DIO) mice through its gut specific anti-inflammatory effect. In addition, cholesterol in western type diets has been suggested to be a driving factor of intestinal inflammation. Therefore, we investigated whether dietary cholesterol can promote intestinal inflammation and attribute to development of type 2 diabetes.

Methods
C57BL/6J mice were fed a low-fat diet (LFD), high fat diet (HFD) or high-fat cholesterol diet (HFCD, 0.25% cholesterol) supplemented with 5-ASA (1500mg/Kg/day) for 12 weeks. Body weight and food intake were recorded weekly. Glucose tolerance test (GTT) was performed on week 11. Fat to lean ratio and gut permeability were measured. Blood, intestines and liver were taken for analysis.

Results
Feeding of HFD or HFCD resulted in obesity and systemic glucose intolerance. HFD or HFCD feeding in comparison with LFD feeding did not affect intestinal permeability as measured by the FITC-dextran assay. Hence, we did not see an effect of administration of the gut anti-inflammatory agent 5-ASA on obesity or systemic glucose intolerance.

Conclusions
Our results indicate that feeding of a HFD or HFCD does not directly affect intestinal barrier function.
Introduction

Obesity is associated with a series of metabolic complications, including dyslipidemia, insulin resistance (IR), type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD). As current preventative and pharmacological therapeutic approaches have had limited success so far, it is evident that new strategies for treating these diseases are urgently needed.

Studies in the past have left little doubt about the involvement of low-grade chronic inflammation in the mechanisms underlying IR and T2D. More recently, several studies have indicated a critical role for the gut in the development of low-grade chronic inflammation in IR and T2D. Consumption of high fat diets promotes intestinal inflammation and triggers disruption of tight junctions proteins leading to increased intestinal permeability and leakage of endotoxins into the systemic circulation (Cani et al., 2007; Ding et al., 2010; Luck et al., 2015). Leakage of endotoxins into the systemic circulation consequently attributes to low-grade-systemic inflammation and type 2 diabetes. Targeting gut inflammation with 5-aminosalicylic acid (5-ASA) improves systemic metabolic parameters and insulin resistance during high-fat feeding in mice, thereby highlighting the importance of HFD-induced intestinal inflammation in the development of metabolic disease (Luck et al., 2015).

Recent studies in zebrafish have indicated that cholesterol in high fat diets is a driving factor for intestinal inflammation (Progatzky et al., 2014). Uptake of dietary cholesterol via NPC1L1 into intestinal epithelial cells leads to inflammasome activation and production of IL-1β leading to the recruitment of neutrophils and macrophages into the gut (Progatzky et al., 2014). Altogether this indicates that dietary cholesterol may contribute to the development of metabolic syndrome by promoting intestinal inflammation leading to leakage of endotoxins into the systemic circulation. To understand the role of dietary cholesterol on intestinal barrier function in the development of the metabolic syndrome, we carried out a set of metabolic experiments in mice fed a low-fat diet (LFD), a high-fat diet (HFD) or a high-fat cholesterol diet (HFCD, 0.25% cholesterol) supplemented with the gut anti-inflammatory agent 5-ASA (1500mg/Kg/day) for 12 weeks. Our data indicate that feeding of a HFD or HFCD for 12 weeks did not lead to increased intestinal permeability, consequently no effects of 5-ASA treatment on development of insulin resistance were observed.
Methods

Mice and treatment
All experiments were performed according to Dutch law and approved by the Ethical Committee for Animal Experiments of the University of Groningen, the Netherlands. Experiments were carried out on male C57BL/6J mice purchased from Charles River (France). Mice were housed separately in Individual Ventilated Cages and maintained on a 12-hour light/12-hour dark cycle with ad libitum access to food and water. Mice were randomized in 6 groups of 10 mice each. Mice were fed either low-fat diet (LFD; 10% kcal fat, Research Diets), LFD 5-ASA, high-fat diet (HFD; 60% kcal fat, 0.02% cholesterol, Research Diets), HFD 5-ASA, HFD supplemented with 0.25% cholesterol (HFC; 60% kcal fat, Research Diets) or HFC 5-ASA starting at 10-12 weeks of age for 12 weeks. Mice were sacrificed using cardiac puncture, and blood, liver and intestines were collected for further analysis.

Compounds and metabolic studies
5-ASA powder (Sigma-Aldrich) was mixed into the LFD, HFD and HFC diets at 1,500 mg/kg/day. All diets were prepared fresh weekly to ensure a consistent 5-ASA dose intake throughout the 12-week study. Body weight and food intake was measured weekly. After 11 weeks of dietary intervention, glucose tolerance test (GTT) was performed as previously described (Gruben et al., 2015), and all GTT were performed with a 2g/kg glucose i.p. injection. Blood samples were collected in EDTA-coated tubes before i.p. injection for determination of insulin levels. Samples were spun at 1000g for 10 min at 4°C and insulin concentrations were measured in plasma by ELISA kit (Alpco Diagnostics, Salem, NH). One day prior to sacrifice body composition was analysed using a Minispec Whole Body Composition Analyser (Bruker).

Gut permeability assays
Gut permeability was measured in unfasted mice at time of sacrifice. Mice received 0.6 mg/g bodyweight of FITC-conjugated dextran (Sigma) by oral gavage and blood was collected via cardiac puncture after 4 hr. The concentration of FITC was determined in plasma by fluorometry at 488 nm as described in Chapter 3.
Quantification of plasma and liver lipids
Plasma triglycerides, total cholesterol and free cholesterol were determined at times of sacrifice using commercially available kits (Triglycerides and total cholesterol: Roche; free cholesterol: FS DiaSys, Holzheim, Germany). To measure hepatic lipid content, lipids were extracted from crushed liver samples using Bligh and Dyer’s method (Bligh and Dyer, 1959). Hepatic triglyceride and cholesterol levels were measured using kits that are commercially available (Triglycerides and total cholesterol: Roche; free cholesterol: FS DiaSys).

Quantitative real-time PCR
RNA was isolated using Qiazol reagent, according to the manufacturer’s instructions (Roche). cDNA was synthesized using the Transcriptor Universal cDNA Master kit from Roche, according to their instructions (Roche, Mannheim, Germany). We performed quantitative real-time PCR with a 7900HT PCR system (Applied Biosystems) using SYBR Green Master Mix reagent (Roche). Each sample was run in triplicate and normalized to PPIA as housekeeping gene. We calculated relative fold changes in gene expression normalized to PPIA by the ΔΔCT method using the equation $2^{-\Delta\Delta CT}$. The results are shown as fold changes compared to the HFC group. Primer sequences are listed in Supplemental table 1.

2.7 Statistical analysis
The data were presented as mean ± SEM unless stated otherwise. Comparisons between groups were performed using one-way ANOVA + tukey HSD post-test unless stated otherwise with P < 0.05 considered statistically significant.

Results and Discussion
To understand the role of dietary cholesterol on intestinal health in high-fat diet induced insulin resistance we fed C57BL/6J mice a LFD, HFD, or HFCD with or without the gut anti-inflammatory agent 5-ASA for 12 weeks. Food intake in grams per day was similar between LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD + 5ASA, ensuring that dosage of 5-ASA (1500 mg/Kg/day) was comparable between groups (Figure 1A). Next, we examined the metabolic effects of 5-ASA during feeding of LFD, HFC and HFCD. As expected feeding of HFD or HFCD significantly
increased body weight gain, fat mass and total plasma cholesterol level, whereas triglyceride levels declined (Figure 1B-E). In contrast, these factors were not affected by administration of 5-ASA (Figure 1B-D). Feeding of HFD or HFCD resulted in increased fasting glucose levels and systemic glucose intolerance compared to LFD-fed mice (Figure 2A-B). Treatment of mice with the gut anti-inflammatory agent 5-ASA did however not affect fasting glucose levels or systemic glucose intolerance (Figure 2A, B). This is in contrast to the study by Luck et al, who reported a protective effect of 5-ASA treatment against systemic glucose intolerance.

Figure 1 – Feeding of HFD or HFCD diet promotes obesity and hyperlipidemia

(A) Food intake of mice fed LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD +5-ASA at week 1 and week 11 (B) Body weights of mice fed LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD +5-ASA (C) Fat or lean mass, expressed as percentage of body weight, were analyzed after 12 weeks of LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD +5-ASA in C57BL/6J mice over time, starting at 10-12 weeks of age (D) total cholesterol levels in plasma (E) Triglycerides in plasma after 12 weeks of dietary intervention. P<0.05 (a) with respect to LFD, (b) with respect to LFD 5-ASA; by one-way ANOVA + Tukey HSD post-test.
In addition, we examined the effect of 5-ASA against development of hepatic steatosis. Our study shows that feeding of HFD leads to increased storage of triglycerides and total cholesterol in the liver in comparison with LFD (Figure 2C, D). In addition, feeding of a HFCD in comparison with HFD further increases triglyceride and total cholesterol storage in the liver of C57BL/6J mice (Figure 2C, D). Consistently, histological examination of the liver confirmed increased steatosis following feeding of HFCD vs HFD (Figure 2E). Increased storage of triglycerides and total cholesterol in the liver following addition of cholesterol to a HFD diet is in accordance with literature (Wouters et al., 2008). Interestingly, we also observed a significant reduction in storage of triglycerides in the liver following 5-ASA treatment in HFCD-fed mice, but not after HFD-feeding (Figure 2C). A lack of protection against liver steatosis after HFD-feeding is in contrast with the results of Luck et al who reported a reduction in liver steatosis (Luck et al., 2015). To examine whether differences in triglyceride storage in the liver of HFCD-fed mice also resulted in altered inflammatory levels in the liver we performed qPCR analysis in mice receiving HFCD or HFCD + 5-ASA. Treatment with 5-ASA in HFCD-fed mice leading to a reduction in triglyceride storage did not result in decreased liver inflammation (Figure 2F). To understand the discrepancies between our data and the data of Luck et al in the development of systemic glucose intolerance and hepatic steatosis following administration of 5-ASA we examined whether 5-ASA improved intestinal health during HFD and HFCD feeding. We observed that feeding of a HFD or HFCD for 12 weeks in comparison with LFD did not affect intestinal barrier function as measured by the translocation of FITC-dextran from the gut into the systemic circulation (Figure 3). Consequently, we also did not observe a protective effect of 5-ASA (Figure 3). The absence of an effect on intestinal permeability after feeding of HFD or HFCD is highly surprising and in contrast with previous results from our lab (Supplemental figure 1) and many other groups (Cani et al., 2007; Ding et al., 2010; Luck et al., 2015). This surprising finding indicates that dietary fat or cholesterol does not directly affect the gut barrier function in C57Bl/6 mice. Interaction between HFD-feeding and the gut microbiota has been indicated as a factor promoting intestinal inflammation. Feeding of a HFD in conventional mice promoted intestinal inflammation whereas germfree mice were protected (Ding et al., 2010). In addition, feeding of HFD can lead to the expansion of pathobionts
Figure 2 – The gut anti-inflammatory agent 5-ASA does not protect against systemic glucose intolerance or NAFLD

(A) fasted glucose levels after 12 weeks of dietary intervention (B) oral glucose tolerance test (C) triglyceride levels in liver in µmol/g (D) total cholesterol levels
such as Bilophila Wadsworthia and Attaching Invading Eschericia Coli (AIEC), consequently promoting intestinal inflammation (David et al., 2014; Devkota et al., 2012; Martinez-Medina et al., 2014). Thus absence of specific pathobionts in our mice during feeding of HFD or HFCD may prevent development of intestinal inflammation and increased intestinal permeability. Luck et al showed induction of intestinal inflammation leading to increased intestinal permeability and leakage of endotoxins promoting low-grade systemic inflammation and insulin resistance following feeding of a HFD. HFD- or HFCD-feeding did however not result in decreased barrier function in our study, providing a plausible explanation of why administration of 5-ASA did not result in protection against systemic glucose intolerance and NAFLD in our studies.

**Conclusion**
In conclusion, our data suggest that consumption of HFD or HFCD does not cause abrogated intestinal barrier function, indicating that dietary fat or cholesterol do not directly affect intestinal barrier function.

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**Conflict of interest**
The authors declare no conflict of interest.

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*in liver in \( \mu \text{mol/g} \) (E) hematoxylin and eosin slides of liver (F) inflammatory gene expression in liver of HFCD and HFCD + 5-ASA fed mice. Data are presented as means ± SEM, \( n = 10 \) mice. \( P<0.05 \) (a) with respect to LFD, (b) with respect to LFD 5-ASA (c) with respect to HFD (d) with respect to HFD 5-ASA; by one-way ANOVA + Tukey HSD post-test.*
Figure 3 – HFD or HFCD feeding does affect intestinal barrier function in C57BL/6J mice

FITC-dextran in vivo permeability assay in mice fed LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD +5-ASA. Data are presented as means ± SEM, n = 10 mice.
Supplemental figure 1

FITC-dextran in vivo permeability assay in Ldlr-/- mice fed a chow diet, HFC for 8 weeks or HFC diet for 13 weeks. Ldlr-/- chow n=10, 8 weeks HFC n=10 13 weeks HFC n=9. Data are presented as means ± SEM, (a) P<0.05 (a) with respect to Ldlr-/- Chow; by unpaired two-tailed Student's t-test.
References


