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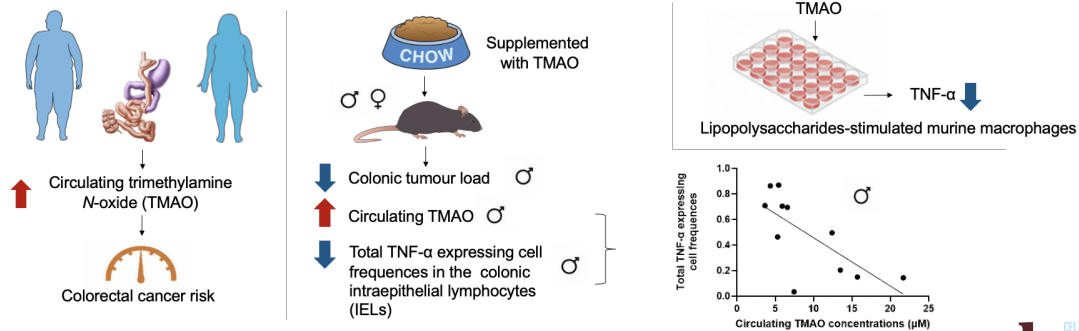
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Methods and Outcome



Summary Circulating TMAO confers protection against colonic tumor growth in male mice, likely through the down-regulation of TNF- α -expressing cell frequencies in the colonic IELs.

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Trimethylamine N-oxide, a bariatric surgery-associated host-microbial co-metabolite, reduces colonic tumorigenesis in a sex-dependent manner

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20 **Summary**

21 Although the association between bariatric surgery and colorectal cancer (CRC) risk remains
22 controversial, Roux-en-Y gastric bypass has consistently been shown to elevate circulating TMAO
23 levels, which have been associated with higher CRC risk. However, our findings reveal that dietary
24 TMAO supplementation results in greater systemic retention of TMAO in male compared with female
25 mice. This higher circulating TMAO level confers protection against colonic tumor growth in a
26 sex-dependent manner, likely through the downregulation of TNF- α -expressing cell frequencies in
27 colonic intraepithelial lymphocytes.

Abstract

Background & Aims

Trimethylamine *N*-oxide (TMAO) is a host-microbial co-metabolite that significantly increases following Roux-en-Y gastric bypass (RYGB). While TMAO is associated with cardiovascular diseases, its role in colorectal cancer (CRC) remains unclear.

Methods

Fabp1Cre;Apc^{15lox/+} mice, a genetically altered CRC model, together with murine macrophages and human colonic cancer cells (HCT-116) were used to investigate TMAO impact on colonic tumorigenesis.

Results

TMAO supplementation significantly reduced the colonic tumor load in male, but not female mice. Consistently, dietary TMAO resulted in higher retention of circulating TMAO in males, which was significantly and inversely correlated with tumor loads. Furthermore, TNF- α -expressing cell frequencies in the colonic intraepithelial lymphocytes (IEL) were significantly lower in TMAO-supplemented male mice compared to controls, suggesting that circulating TMAO could play a protective effect against colonic tumor growth via down-regulation of TNF- α -expressing cells. This was supported by *in vitro* observations that TMAO reduced lipopolysaccharides (LPS)-stimulated TNF- α production from macrophages. TMAO exerted no effects on cell proliferation (Ki67) and DNA damage (γ H2AX) of HCT-116 cells.

Conclusion

Our study leads us to conclude that higher retention of circulating TMAO has a protective effect against CRC in a sex-dependent manner, highlighting the importance of understanding the complex relationship amongst the concentrations of host-microbial cometabolite TMAO, its systemic circulation, and its biological function in modulating CRC risk.

56 **Keywords**

57 Weight loss surgery • bacterial metabolism • microbiota • metabolism • colon cancer risk • DNA
58 damage • inflammation • cytokines

59

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60 Introduction

61 Bariatric surgery, including Roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy, induces
62 sustained weight loss and improves obesity-related co-morbidities[1]. However, several cohort
63 studies highlighted the potential association between bariatric surgery and increased colorectal
64 cancer (CRC) risk[2, 3]. Furthermore, RYGB is associated with a significant shift in the gut microbiota
65 towards enrichment of members from the *Enterobacteriaceae* family and increased host-microbial
66 cometabolites, such as trimethylamine *N*-oxide (TMAO) in biofluids[4-7]. TMAO is produced from
67 oxidation of trimethylamine (TMA) by flavin-containing monooxygenase 3 (FMO3) in the liver[8]. TMA
68 is mainly a bacterial metabolite produced from dietary choline and carnitine, catalyzed by enzymes,
69 such as choline-TMA lyase (*cutC*) and carnitine monooxygenase (*cntA*)[9]. The higher abundance
70 of *cutC* genes has been reported in non-bariatric patients with colorectal cancer (CRC)[10, 11].

71
72 TMAO has been known historically as an osmolyte and it stabilizes structural and functional integrity
73 of proteins[12]. However, there has been much interest in the role of TMAO with respect to
74 inflammation in cardiovascular disease, chronic kidney disease and CRC[13-16]. A genome-wide
75 analysis showed that TMAO was associated with CRC[17] and a positive correlation between plasma
76 TMAO levels and CRC was found in the Women's Health Initiative Observational Study[18]. In a
77 more recent study, higher serum TMAO levels were found to be associated with higher risk of distal
78 CRC, but not overall CRC risk[19]. The empirical mechanistic evidence on the function of TMAO in
79 CRC remains sparse. A rodent-based study showed that TMAO exerted an oncogenic effect by
80 enhancing cell proliferation and angiogenesis in CRC[20]. In contrast, findings on protective function
81 of TMAO have also been reported. Patients with triple-negative breast cancer showed better
82 responses to immunotherapy with higher circulating TMAO concentrations and TMAO levels were
83 higher in tumors with an activated immune microenvironment[21]. Furthermore, intraperitoneal
84 delivery of TMAO or dietary choline supplementation reduced tumor growth in an orthotopic
85 pancreatic ductal adenocarcinoma mouse model and was associated with an immunostimulatory
86 tumor-associated macrophage phenotype and an activated effector T cell response in the tumor

microenvironment[22]. These findings collectively suggested that TMAO potentially regulates immune function and tumor development.

To investigate the causal relationship between TMAO and CRC risk, we employed a *Fabp1Cre;Apc^{15lox/+}* mouse model and a combined methodologies including flow cytometry, histology and metabolomics. *Fabp1Cre;Apc^{15lox/+}* mice better mimic the early-stage development of human sporadic CRC with tumors primarily developed in the large intestine compared to *Apc^{min/+}* mice[23]. Based on the findings from the mouse model, we further explored the impact of TMAO on CRC risk factors, including cell proliferation, DNA damage and inflammation, using macrophages and colon cancer cells.

Results

Dietary supplementation with TMAO significantly reduces colonic tumorigenesis in male mice

Prior to investigating the effects of TMAO in a mouse model, we first quantified circulating levels of TMAO and TMA in patients undergoing bariatric surgery. Bariatric surgery procedures primarily include restrictive techniques, such as laparoscopic gastric banding (LGB), reducing food intake, and the combined restrictive and malabsorptive methods, such as RYGB limiting both food consumption and nutrient absorption [24]. Circulating and urinary TMAO have been found to be elevated following RYGB surgery, in particular patients with type 2 diabetes mellitus (T2DM) [5, 7, 24]. In our cohort of 16 patients (RYGB, n=11; LGB, n=5, Table 1), we quantified circulating TMAO and TMA using UPLC-MS/MS [25] and observed consistent findings of increased concentrations of TMAO in fasting serum samples at 6-month post-RYGB compared to pre-op (Fig. 1a, $p = 0.002$), but this elevation was not noted in the LGB group. Furthermore, TMAO concentrations were significantly higher in the RYGB group compared to LGB group at 6 mo (Fig. 1a, $p = 0.0496$). In contrast, circulating concentrations of TMA did not exhibit statistically significant changes across time points nor between the two surgical groups (Fig. 1b & 1d). Additionally, the increases in TMAO levels following RYGB were observed in groups with or without T2DM (Fig. 1c).

While TMAO has been reported to be positively associated with CRC risk [17, 18], their causal relationship has not been elucidated. We, therefore, investigated the impact of TMAO on colonic tumor development using a *Fabp1Cre;Apc^{15lox/+}* mouse model [23]. This genetically altered model predominantly develops tumors in the large intestine and closely mirrors familial adenomatous polyposis (FAP)-associated CRC [23]. Initially, we performed a baseline study to monitor the intestinal tumor onset and progression in mice fed with normal chow (NC) diet; this study was used to determine the duration of TMAO supplementation and the age of mice for tumor examination. Mice showed clinical symptoms (e.g., blood in the stool) at an average age of 16.75 and 19.57 weeks old for males and females, respectively. Female mice showed a longer survival compared to males ($p=0.0032$) and no statistically significant differences in tumor load, gut length, colon length and spleen weight were observed between males and females (Fig. 2). Considering the sex-related phenotypic differences in tumor development, we selected 13 weeks of age for male mice and 15 weeks for female mice as the end timepoint (T2) to investigate the impact of TMAO on tumorigenesis in the early phase.

Following 1-week acclimatization, mice at the age of 7 weeks old (T0) were fed either NC diet (control group) or NC supplemented with 0.12% (w/w) TMAO for 6 weeks in males and 8 weeks in females (Fig. 3a). In addition, a midpoint (T1) was included to monitor anemia and TMAO levels in urine and plasma (Fig. 3a). While male mice exhibited significantly higher body weight than the female mice, no significant differences observed between the control and TMAO groups of the same sex throughout the experiment (Fig. 3b). The food intake did not differ between male and female mice, nor between control and TMAO groups (Fig. 3c). Intestinal length and tumors were assessed at the end of the experiment; the lengths of the gastrointestinal tract and the colon did not differ between the control and TMAO groups (Figs. 3d & 3e). However, a lower percentage of mice in the TMAO group developed colonic tumors compared to the control group, with a significant decrease observed in males (Fig. 3f). Furthermore, tumor volume, taking both tumor count and size into the consideration, was significantly lower in the TMAO-treated male mice compared to male controls, while this difference was not significant in females (Fig. 3g). This observation was further supported by the histological scores of hematoxylin and eosin (H&E) staining for the colonic tissues and tumors.

Most of male mice presented with no tumors in the TMAO group compared to the control group, unlike in female mice (Fig. 3h). One of the clinical symptoms of these mice with intestinal tumors is anemia, which can be assessed using hematocrit (HTC)[26, 27]. The HTC was significantly lower at T2 compared to T1, indicating the development of anemia (Fig. 3i). These phenotypic observations together allow us to conclude that dietary supplementation with TMAO exerts a protective effect against colonic tumorigenesis in *Fabp1Cre;Apc^{15lox/+}* mice, particularly in males.

Bioavailability of TMAO following dietary supplementation is sex- and supplementation duration-dependent

Given that the dietary supplementation of TMAO significantly reduced the colonic tumor load in male mice, we next explored if bioavailability of TMAO following the dietary supplementation was sex dependent. The TMAO concentrations in the jejunum and ileum luminal contents were significantly higher in the TMAO group compared to the control group in both male and female mice (Fig. 4a). However, this increase was not observed in the colon luminal content, suggesting that the absorption of dietary TMAO mainly occurs in the small intestine. No differences in the TMAO concentrations in the intestinal lumens were noted between male and female mice. Furthermore, no statistically significant differences in the intestinal luminal TMA concentrations were observed (Fig. 4b).

While circulating TMAO concentrations in female mice are intrinsically higher than the males as previously reported[28] and as observed in our study (Fig. 4c, T0 before the dietary supplementation), supplementation with TMAO for 3 or 4 weeks resulted in their significant increases in male and female mice, respectively (Fig. 4c, T1). However, the TMAO supplementation did not induce significant changes in TMA concentrations in the plasma at T1 (Fig. 4d). Following the supplementation with TMAO for 6 and 8 weeks (T2) in male and female mice, respectively, plasma TMAO remained elevated at T2 in males but not females (Fig. 4c, T2). To further investigate this discordant trend, we measured TMAO excretion in the urine, where we observed that TMAO supplementation resulted in significantly higher concentrations of TMAO in females, but not in males (Fig. 4e). The ratios of plasma to urinary TMAO levels were significantly higher in TMAO supplemented male mice compared to the controls; however, this pattern was not observed in female

mice (Fig. 4g). Notably, male mice showed higher TMA excretion in TMAO-treated group compared to the controls at both T1 and T2 (Fig. 4f), which led us to conclude that there was an enhanced retro-conversion of TMAO to TMA through the gut bacterial activity[29] following TMAO supplementation in males.

We next explored the correlation between the circulating concentrations of TMAO at T1 and T2 and the colonic tumor volume. Circulating TMAO levels measured at T1 and T2 were negatively correlated with tumor volume in male mice, with a statistical significance ($p = 0.0269$, $r = -0.4608$) at T2 (Fig. 4h). However, this correlation was not evident in females (Fig. 4i). Additionally, TMAO supplementation did not induce significant shifts in the ^1H Nuclear Magnetic Resonance (NMR) spectroscopy-based global metabolic profiles of urine (Fig. 5a-d), plasma (Fig. 5e-g) and gut luminal contents (Fig. 5i-k). However, it was noted that the metabolome of luminal contents from the jejunum, ileum and colon were distinct (Fig. 5h).

These findings let us to conclude that absorption of dietary TMAO mainly occurred in the small intestine in both male and female mice. While TMAO supplementation increased the circulating TMAO levels in both male and female mice at the midpoint T1, the retention of TMAO at the endpoint T2 was higher in male compared to female. This higher retention of TMAO was found to be significantly inversely associated with the colonic tumor load in males.

Dietary supplementation of TMAO altered the colonic immune landscape and significantly lowered percentages of TNF- α -expressing intraepithelial lymphocytes in male mice

To further investigate the potential mechanism through which TMAO supplementation induced anti-tumorigenic responses in *Fabp1Cre;Apc^{15lox/+}* mice, we assessed the landscape of immune cell subsets within the colonic intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) using flow cytometry. As we focused on the effect of TMAO on CRC risk, we analyzed IEL and LPL instead of tumors, which represent tumor microenvironment-related immune responses. The initial observation revealed a higher percentage of the total CD45⁺ cell in IEL of male mice receiving TMAO supplementation compared to the controls, although the statistical significance was not reached (Fig.

6a). We concluded that there was a potential increase in immune cell infiltration, indicating a heightened immune response, in response to elevated concentrations of TMAO. In both control and TMAO groups, CD8⁺ T cells and $\gamma\delta$ T cells dominated in the IEL, while myeloid cells, CD4⁺ T cells, and Natural Killer (NK) cells were predominant in the LPL (Fig. 6b). When comparing the TMAO group to the controls, CD8⁺ T cell frequencies were higher in LPL of both male and female mice. In contrast, group 1 innate lymphoid cell (ILC) frequencies were higher in both IEL and LPL from female mice fed with TMAO supplementation compared to the control group, but this trend was not observed in male mice (Fig. 6c). Furthermore, TMAO supplementation in male mice increased the frequencies of NK cells in both IEL and LPL, whereas in females, it led to a decrease (Fig. 6c). In addition, the ratio of CD8⁺ to FoxP3⁺ was calculated since it has been reported as an indicator of the balance between cytotoxic T cells and regulatory T cells (Tregs). A high ratio of CD8⁺ to FoxP3⁺ in the tumor microenvironment has been associated with improved outcomes in different types of cancer, such as breast cancer and CRC [30, 31]. In our study, we observed a slight increased CD8⁺/Foxp3⁺ ratio in the colonic mucosa of TMAO-fed male mice (Fig. 6d), potentially suggesting a shift toward a more cytotoxic and less immunosuppressive immune profile. This balance suggests that TMAO may enhance local anti-tumor immunity by promoting effector T-cell activity while limiting regulatory T-cell-mediated suppression.

Following the compositional analysis of immune cell subsets, we investigated the TMAO-induced changes at the functional level and these included CD107a, Granzyme B (GrB), Interferon-gamma (IFN- γ), Interleukin 17 (IL17), natural killer group 2 member D (NKG2D), programmed cell death protein 1 (PD1), and tumor necrosis factor alpha (TNF- α). We observed that TMAO supplementation in male mice significantly lowered percentages of TNF- α -producing CD4⁺ T, CD8⁺ T, $\gamma\delta$ T and NK cells in the IEL (Fig. 6e). In contrast, dietary TMAO did not affect TNF- α -expressing in innate immune cells, such as CD11b⁺Ly6C⁺Ly6G⁻ monocytic-myeloid-derived suppressor cells (M-MDSCs), CD11b⁺Ly6C⁻Ly6G⁺ polymorphonuclear-myeloid-derived suppressor cells (PMN-MDSCs), CD11b⁺F4/80⁺ macrophages, group 1 ILCs and group 3 ILCs, nor in total CD3⁺ T cells within the colonic IEL (Fig. 7a & 7b). Furthermore, TMAO supplementation reduced the percentage of GrB-

producing $\gamma\delta$ T cells in the IEL of male, but not in female mice (Fig. 6f). Total TNF- α -expressing cell frequencies were significantly and negatively correlated with circulating TMAO concentrations in males ($p = 0.0098$, $r = -0.7545$, Fig. 6g) but not in females (Fig. 6f). These changes observed in the colonic IEL were not noted in the colonic LPL (Fig. 7c-e). The immune profiles of LPL and IEL from the duodenum-jejunum and the ileum showed no evident differences between control and TMAO groups (Fig. 8). However, it was noted in the duodenum-jejunum LPL that the Group 1 ILCs ratio between TMAO and control mice was higher in female (ratio = 2.38) compared to males (ratio = 0.64, Fig. 8f), but this trend was reversed in the ileum (female, ratio = 0.09 and male, ratio = 6.52, Fig. 8l).

Collectively, our data demonstrated that dietary supplementation with TMAO reduced the percentages of TNF- α -expressing CD4⁺ T cells, CD8⁺ T cells, $\gamma\delta$ T and NK cells in the colonic IEL of male mice, favoring a reduced inflammatory environment in the colon to reduce tumorigenesis.

TMAO reduces TNF- α levels in lipopolysaccharide (LPS)-stimulated murine macrophages

Following our observation that dietary supplementation of TMAO reduced TNF- α -expressing immune cell subsets in the colonic IEL in male mice, we investigated the direct impact of TMAO on TNF- α secretion from LPS-stimulated murine macrophages (RAW264.7 cell line originally established from a male mouse). We first confirmed that TMAO and TMA at the concentration ranges of 5 μ M – 500 μ M and 5 μ M – 150 μ M, respectively, did not induce cytotoxic effects (Fig. 9a-d). We next chose physiological circulating concentrations of TMAO (50 μ M) and TMA (150 μ M) observed in the male mice at T2 (Fig. 4c) to prime cells for 24 hours and exposed the cells to LPS (10 ng/ml) for 16 hours. Both TMAO and TMA significantly suppressed TNF- α secretion (Fig. 9e), which is consistent with our observations *in vivo*. Furthermore, TMAO exposure at a concentration of 50 μ M for 24 hours to human colon cancer HCT116 cells did not induce cell proliferation (Ki67⁺) and genotoxicity (γ H2AX) (Fig. 10), and we suggest that TMAO did not increase colonic cancer risk factors.

Discussion

Consistent with previously published findings[7], we observed that TMAO circulating concentrations significantly increased 6 months post-RYGB surgery, but not after LGB. Although it was reported that circulating TMAO increased more than twofold in patients with T2DM after RYGB surgery, but not in patients without T2DM[5], we found that both groups of patients exhibited significant increases, with a higher degree observed in patients with T2DM. In contrast, TMA levels remained similar before and after the surgery. In a long-term follow-up study, circulating levels of TMAO have remained significantly higher 8 years post-RYGB[7].

In the current study, we directly demonstrated the protective effects of dietary TMAO against colonic tumor development in a sex-dependent manner using a genetically altered murine model, *Fabp1Cre;Apc^{15lox/+}* mice, which develops colonic tumors and better mimics the human sporadic CRC. We further reported that plasma TMAO concentrations, resulted from dietary TMAO supplementation, were negatively correlated with the tumor load in male, but not female mice. This protective role of TMAO has not previously been reported in CRC, but lower serum levels of TMAO were observed in patients with pancreatic cancer[32]. Furthermore, TMAO was recently shown to reduce tumor growth in mice with orthotopic pancreatic ductal adenocarcinoma (PDAC)[22]. Mirji and colleagues reported that delivering TMAO intraperitoneally or via a dietary supplement to orthotopic PDAC-bearing mice reduced their tumor growth, which was associated with an immunostimulatory tumor-associated macrophage phenotype, and activated effector T cell response in the tumor microenvironment[22]. Another study demonstrated that triple-negative breast cancer (TNBC) patients with higher plasma TMAO achieved better responses to immunotherapy and TMAO induced tumor cell pyroptosis by activating protein kinase R-like endoplasmic reticulum kinase (PERK) and enhanced CD8⁺ T cell-mediated antitumor immunity[33]. These findings are in line with our observation that higher circulating levels of TMAO in males exert a protective role against colonic tumor development *in vivo* and provide a rationale to explore the immune and inflammatory effects of TMAO in CRC. However, a recent study in *Apc^{min/+}* female mice reported that orally administered TMAO promoted intestinal tumorigenesis by suppressing the farnesoid X receptor-fibroblast growth

factor 15 (FXR-FGF15) axis. These findings indicate that effects of TMAO on CRC is context-dependent, varying with experimental model, sex, and exposure conditions[34].

We revealed a new role of dietary TMAO in modulating colonic immune subsets; dietary TMAO significantly reduced frequencies of TNF- α expressing CD4⁺ and CD8⁺ T cells, and $\gamma\delta$ T and NK cells in the colonic IEL of male mice. TNF- α is a known pro-inflammatory cytokine that is associated with tumor promotion by bridging inflammation and carcinogenesis[35]. Specifically, it stimulates the production of other pro-inflammatory cytokines, such as IL-6 and IL-8, which contribute to the inflammatory microenvironment associated with CRC[36]. Furthermore, we found that the circulating TMAO concentrations were negatively associated with the total TNF- α expressing immune populations and exposure of TMAO to murine macrophage RAW264.7 cells significantly reduced LPS-stimulated TNF- α production. From this evidence we propose that higher circulating TMAO concentrations could suppress tumorigenesis through reducing TNF- α -expressing immune subsets. Blocking TNF- α signaling or using TNF- α knockout mice markedly suppresses intestinal tumor development[37, 38]; hence, TNF- α signaling may be a promising target for reducing obesity-associated CRC risk[37]. Following Roux-en-Y gastric bypass (RYGB), increased TMAO levels may exert a beneficial immunomodulatory effect; however, this possibility requires further investigation in cohorts of patients who have undergone RYGB.

Our rodent experiment with dietary TMAO supplementation also showed compelling evidence that the dietary TMAO was mainly absorbed in the jejunum and ileum, circulating TMAO levels were affected by its urinary excretion, and the duration of supplementation dynamically affected the circulating TMAO levels in female mice. We revealed that urinary excretion of TMAO was not affected by dietary supplementation in male mice, but significantly increased in females, as a result, a higher retention of blood TMAO in male mice compared to females was observed. It was estimated that 50% of ingested TMAO remain unchanged and excreted from urine and the other 50% undergoes retro-conversion to TMA and re-oxidized to TMAO before urinary excretion[39]. We observed an

increase in circulating TMA in male mice at the final time point, but not in females, which could be explained by previously reported higher FMO3 activities oxidizing TMA to TMAO in females[40, 41].

In summary, our novel findings suggested that circulating TMAO played a protective effect against colonic tumor growth through down-regulating TNF- α -expressing cell frequencies. This study contributes to the growing interest in CRC risk following bariatric surgery and the role of TMAO in modulating CRC risk, and it provides a new perspective on the relationship between dietary TMAO intake and circulating TMAO levels, highlighting the importance of circulating TMAO retention in CRC prevention.

Limitations of the study

While our study provided evidence supporting the causal relationship between TMAO and CRC risk, several limitations can be addressed in the future studies. We quantified the circulating TMAO and TMA levels in patients at 6 months post RYGB or LGB. Long-term follow up studies with longitudinal collection of paired urine and blood samples will be insightful for understanding TMAO circulation and retention post bariatric surgery. Nevertheless, our findings highlighted the importance of TMAO retention in the blood in CRC development. The effect of TMAO on colonic tumorigenesis was assessed using *Fabp1Cre;Apc^{15lox/+}* mice in the current study. We chose this model as it has been shown to better mimic the human sporadic CRC compared to *Apc^{min/+}* mice[23]. As we highlighted a role of TMAO on down-regulating TNF- α -expressing cell frequencies in colonic IEL, it would be interesting to further investigate the role of TMAO in TNF- α knock-out mice. TNF- α knock-out has been reported to attenuate the high-fat diet-associated increase in intestinal tumorigenesis[37]. Female mice generally exhibit higher circulating and urinary TMAO and lower TMA levels compared to males. In our study, we monitored the temporal changes in TMAO and TMA levels over a 7-week experiment, which prompted us to conclude that male mice demonstrated higher retention of circulating TMAO. Here, we cannot preclude the potential effects of TMA on intestinal tumorigenesis and the downregulation of TNF- α -expressing cell frequencies in colonic IEL. Further investigation is warranted to distinguish the separate effects of TMAO and TMA.

341

342 **Materials and Methods**

343 **Patient cohort**

344 A total of 16 patients (undergoing laparoscopic gastric banding (LGB) n=5 or RYGB n=11) were
345 recruited from Emory University, USA (IRB #333-2002). Serum samples were collected at pre-
346 operative (pre-op) and 6 months after surgery (post-op), as previously detailed [24].

347

348 **Fabp1Cre;Apc^{15lox/+} mouse experimental design**

349 The animal study was conducted in accordance under the UK Home Office Animals Scientific
350 Procedures Act 1986 and the EU Directive 2010, with a UK Home Office project license of PPL
351 P9718F9C8. Fabp1Cre;Apc^{15lox/+} mice[23] were generated by crossing Apc^{15lox/15lox} (JAX stock
352 #029275) with homozygous Fabp1 Cre mice in the animal facility at Imperial College London (London,
353 UK). Mice were maintained under specific pathogen-free conditions on a 12-hour light-dark cycle at
354 a temperature of 21±1°C. The health status was monitored daily to ensure their welfare.

355

356 A preliminary baseline study was conducted to determine the onset of intestinal tumorigenesis and
357 establish comparable experimental endpoints between sexes in Fabp1Cre;Apc^{15lox/+} mice. We
358 observed that males began developing tumors two weeks earlier than females (at 13 weeks old for
359 males and 15 weeks old for females). Therefore, the mice were sacrificed at these ages in the
360 following TMAO experiment.

361

362 A total of 46 male and 48 female 6-week-old Fabp1Cre;Apc^{15lox/+} mice were included in the TMAO
363 supplementation experiment. 6-week-old mice were acclimatized in the facility for one week with free
364 access to water and normal chow (NC) diet (Product code 1535-00, Ssniff, Germany). Following
365 acclimatization, mice were randomized into two groups: control group (controls) fed on NC, and the
366 TMAO group fed on the same NC, but containing 0.12% (w/w) TMAO (Product code S9468-P020,
367 Ssniff, Germany). To minimize potential bias, mice from the same breeding pairs with comparable
368 body weights were allocated to different treatment groups. This supplementation dose of 6 mg TMAO

per day ($0.12\% \times 5$ g of daily food intake (equivalent to 15 kcal/day) has previously been used to study impact of TMAO on cardiovascular diseases [42, 43]. The dose corresponds to an individual's daily intake of 800 mg of TMAO per 2000 kcal (a factor of 133.3 in calorie intake) and this intake level is equivalent to consuming approximately 200 g of red bream fish [44, 45]. At the age of 7 weeks post acclimatization, male and female mice were subjected to 6- and 8-week supplementation of TMAO in their diet.

Blood samples were collected by tail venipuncture at baseline (T0; after acclimatization, before the group assignment), midpoint (T1; at the middle period of dietary intervention, age of 10 weeks for males and 11 weeks for females) and endpoint (T2, at the end of dietary intervention, age of 13 weeks for males and 15 weeks for females). Blood was collected into heparin-coated capillary tubes and centrifuged at 11,200 *g* for 3 mins to obtain plasma. Hematocrit (HCT) was calculated as the proportion of packed red blood cells within the total blood column to assess anemia [46]. Urine and fecal samples were collected for metabolomics studies at T0, T1 and T2. Biofluid samples were stored in a -80°C freezer pending analysis.

Mice were sacrificed at the age of 13 and 15 weeks for male and female, respectively, to collect the gut luminal contents. Researchers were blind to the mouse groups. After the removal of the cecum, the small intestine was divided into three equal parts: duodenum, jejunum, and ileum. After removing the remaining fat, the content from each part of the intestine was collected. The gut was opened carefully to avoid cutting through tumors and tumors were counted and the size was measured. The tumor volume was calculated using the following formula: $(4/3) \times \pi \times (\text{diameter of each tumor}/2)^3$.

Histological analysis of intestinal tumors

Histological analysis of tumor samples involved fixation in 4% v/v formaldehyde solution, followed by staining with H&E. This staining method allows for visualization of tissue structure, cell distribution, and morphological changes. A blind evaluation of the stained slides was carried out by a pathologist. Tumor scoring was based on the following criteria: Score I: tubular adenoma with low-grade dysplasia; Score II: tubular adenoma with high-grade dysplasia; Score III: tubular adenoma

with high-grade dysplasia or well-differentiated adenocarcinoma; and Score IV: tubular adenoma with severe dysplasia or moderately differentiated adenocarcinoma. Representative images of H&E-stained sections of colon tumors with Score I to IV are shown in Figure 3h.

¹H NMR spectroscopy-based metabolic profiling of urine, plasma and gut contents

¹H NMR spectroscopy was applied to generate global metabolic profiles of urine, plasma and gut content samples [47]. Urine and plasma samples were thawed at room temperature (21±1°C) and spun at 20,000 *g*, 4°C for 10 minutes. For Urine analysis, a total of 25 µL of the supernatant from each sample was mixed with 34 µL deuterium oxide (D₂O, purity 99.99%, Merck) and 6 µL of potassium phosphate buffer [47]. The urine mixture was briefly spun and 60 µL was transferred to an NMR tube with an outer diameter of 1.7 mm using an automatic syringe pipette (SGE™ eVol™ Handheld Automated Analytical Syringe). For plasma analysis, a total of 25 µL of the supernatant from each sample was mixed with 15 µL deuterium oxide (D₂O, purity 99.99%, Merck) and 40 µL of plasma potassium phosphate buffer [47], and 60 µL of the mixture was transferred to a 1.7-mm NMR tube.

Approximately 150 mg of gut content from the jejunum, ileum and colon were weighed and two portions of HPLC water (e.g., adding 200 µL to 100 mg of content) were added to the sample. The pellets were vortexed for 5 minutes and centrifuged at 20,000 *g* for 10 minutes at 4°C. A volume of 180 µL of the supernatant was transferred to a new 1.5 mL microcentrifuge tube containing 20 µL of the potassium phosphate buffer [47]. The mixture was vortexed for 5 seconds and briefly spun. A total of 180 µL of the supernatant was transferred to a 3 mm NMR tube for ¹H NMR spectroscopic analysis.

¹H NMR spectra of medium, urine and gut content samples were obtained using a 600 MHz ¹H NMR spectrometer Bruker AVANCE III (Bruker; Rheinstetten, Germany), operating ¹H frequency at 600.13 MHz at a temperature of 300 K for urine and gut content, and 310 K for plasma. A standard NMR pulse sequence was applied (one-dimensional (1D) nuclear Overhauser enhancement spectroscopy (NOESY) - preset (noesypr1d): relaxation delay-90°-t₁-90°-t_m (mixing time)-90°-acquisition) to

acquire one-dimensional (1D) spectral data. The spectral width is 20 ppm and 4 dummy scans were used. A total of 64 scans were collected into 20k data points for urine, gut content, and plasma. Phase correction, baseline correction and calibration to the singlet from TSP at 0.00 ppm were manually performed in TopSpin 3.1 (Bruker, Germany). ¹H NMR spectra were imported to MATLAB (R2014a) software. The peaks regions of TSP ($\delta^1\text{H}$ -0.2-0.2) and water ($\delta^1\text{H}$ 4.55-4.80) were removed. The probabilistic quotient method was applied to normalize the remaining spectra[48]. Principal component analysis (PCA) was constructed on the mean-centered and unit variance-scaled data in SIMCA (13.0).

TMAO and TMA in intestinal contents and urine were identified using Chenomx NMR suite 8.3 software (Chenomx Inc. Edmonton, Alberta, Canada). The peaks of TMA (singlet at 2.88 ppm, range from 2.8760 ppm to 2.8910 ppm), TMAO (singlet around 3.26 ppm, range from 3.2550 ppm to 3.2800 ppm) and TSP (singlet at 0 ppm, range from -0.2000 ppm to 0.2000 ppm) were integrated from gut content and urinary spectra, and the concentration of TMA and TMAO were calculated based on the concentration of TSP using the following equation.

$$TMAO\ concentration = \frac{\Delta integrals\ of\ TMAO\ peak}{\Delta integrals\ of\ TSP\ peak} * TSP\ concentration$$

Quantification of TMAO and TMA using a targeted UPLC-MS/MS

TMAO and TMA concentrations in patient serum and mouse plasma were quantified in patient serum samples and mouse plasma samples were quantified using a previously established targeted ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method [25]. Briefly, 10 μL of the sample was mixed with 10 μL of the internal standard solution containing TMA- d_9 hydrochloride (Cayman. Ann Harbor, MI, USA) and TMAO- d_9 (Toronto Research Chemicals, North York, Ontario, Canada) at 50 μM and 160 μM , respectively, in acetonitrile with 10 mM hydrochloric acid. The mixture was briefly vortexed immediately before adding 45 μL of ethyl bromoacetate (Sigma-Aldrich, Steinheim, Germany) solution (15 mg/mL, 1% (v/v) ammonium hydroxide in acetonitrile). The mixtures were incubated at room temperature for 30 minutes for derivatization, which was subsequently stopped by adding 185 μL of acetonitrile/water (95:5, v/v)

solution. The mixtures were centrifuged at 18,000 g for 10 minutes at 4°C and 100 µL of the supernatant was transferred to a LC-MS glass vial and mixed with 50 µL of 1 M sodium heptanesulfonate solution (Sigma-Aldrich, Steinheim, Germany) in water.

Separation of TMAO, TMA and labelled internal standards was obtained using a UPLC-MS/MS system (Waters, Milford, Massachusetts, United States) and a gradient separation combining an aqueous mobile phase A (0.1% formic acid in HPLC grade water, v/v) and organic mobile phase B (0.1% formic acid in acetonitrile, v/v), through a reversed phase HSS T3 2.1 x 150 mm column (Waters, Milford, Massachusetts, United States) with a particle size of 1.8 µm. All solvents were HPLC grade (Fisher scientific) and formic acid purity was 98-100% (Merck). The starting separation conditions were 15% B and held for 1.5 minutes, increased to 55% B for 1.5 minutes, further increased to 99.9% B and held for one minute, before gradually returning to the initial conditions within 2 minutes. The total running time was 6 mins at a flow rate of 0.35 mL/min. The injection volume was 2 µL and the column temperature was maintained at 40°C. Elution time for TMAO and internal standard was 2.51 mins, and 2.71 minutes for derivatized TMA and TMA-D₉. The mass spectrometer conditions were performed at positive electrospray ionization (ESI+) mode. The capillary voltage was set at 1.5 kV. The desolvation temperature was 350°C, the desolvation gas flow was 800 L/H and the cone temperature was 150°C. Multiple reaction monitoring (MRM) ion pair masses for TMAO were 76→59 and 85→68 for TMAO-d₉. Derivatized masses for TMA and TMA-d₉ were 146→118 and 155→127, respectively.

Concentrations of TMAO and TMA in the samples were calculated based on the integrated peak area ratios obtained from MassLynx software (V2.1, Waters Corporation), which were proportional to the concentrations. A linear regression equation was constructed using Excel by plotting the calibration curve concentrations (ranging from 0.001 µM to 100 µM) for each compound on the x-axis and the peak area ratios for TMAO or TMA standards and their labeled counterparts on the y-axis. The concentrations of TMAO and TMA calculated were corrected for the corresponding sample dilution factor.

Lymphocyte isolation from intestinal tissues

Intestinal tissues were collected from 6 mice per group per sex for lymphocytes isolation. Lamina propria (LPL) and intraepithelial lymphocytes (IEL) were isolated separately from the duodenum–jejunum, ileum, and colon. For IEL isolation, tissues were incubated in Hank's balanced salt solution (HBSS; Sigma-Aldrich) containing 1 mM dithiothreitol (DTT, Sigma-Aldrich), 2% (v/v) fetal calf serum (FCS, Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich), and 100 µg/mL streptomycin (Sigma-Aldrich) for 15 min at 37°C with rotation. Tissues were then transferred to HBSS containing 1 mM EDTA (Sigma-Aldrich), 2% (v/v) FCS, penicillin, and streptomycin and incubated for a further 15 min at 37°C. The resulting cell suspension was passed through a 100-µm cell strainer and collected on ice. The EDTA extraction was repeated once, and the two IEL fractions were pooled in a 50 mL Falcon tube.

Following IEL isolation, the remaining tissue left on the cell strainer was cut into small pieces and transferred into a well on a 6-well plate. A total of 7 mL of RPMI buffer (Thermo Fisher Scientific) containing 5% v/v FCS, 100 U/mL Collagenase VIII and (Sigma-Aldrich) and 50 U/ml DNase (Sigma-Aldrich) was added into each well to isolate LPL. The plate was placed on a shaker at low speed (100 rpm) for 30 mins and the solution containing tissue pieces in each well were transferred onto a 100-µm cell strainer to collect the flow-through containing LPL in a 50 mL Falcon tube. The strainer and digestion vessel were rinsed with PBS containing 0.5% (v/v) bovine serum albumin (BSA) and EDTA, and the resulting suspension was combined with the LPL fraction. The tubes containing IEL and LPL suspensions were centrifuged (10 min, 4°C, 500 g) and resuspended in 1 mL of PBS for staining.

Mesenteric lymph nodes (MLN) were harvested and used as controls. MLNs were mechanically dissociated through a 100-µm cell strainer in RPMI medium supplemented with 5% (v/v) FCS. Cell suspensions were centrifuged at 500 × g for 5 min at 4°C and resuspended in PBS for subsequent flow cytometric analysis.

Flow cytometry and staining

A 24-color spectral flow cytometry panel was used to characterize intestinal immune cell populations. The panel included markers for T cells, B cells, NK cells, innate lymphoid cells (ILCs), macrophages, and myeloid-derived suppressor cells, together with functional and intracellular markers including IFN- γ , TNF- α , IL-17, granzyme B, T-bet, ROR γ t, and Foxp3 (Table 2). Fluorescence-minus-one (FMO) controls were included for selected markers to facilitate consistent gating.

Prior to staining, aliquots of IEL, LPL, and MLN cell suspensions were pooled to generate unstained and FMO controls. Cells were incubated with Fc receptor-blocking reagent (eBioscience) and stained with Zombie UV™ Fixable Viability Dye (BioLegend) for 20 min. Cells were subsequently stained with the extracellular antibody cocktail for 30 min and washed twice. For intracellular staining, cells were fixed and permeabilized using the Transcription Factor Fixation/Permeabilization Kit (eBioscience) for 30 min, followed by staining with intracellular antibodies for 30 min and washing.

Samples were acquired on a Cytex Aurora spectral flow cytometer using SpectroFlo software (Cytex Biosciences). Spectral unmixing was performed using appropriate single-stained reference controls, and FMO controls were used to define gating boundaries where required. Flow cytometry data were analyzed using FlowJo v10 (BD Biosciences). After auto-compensation during acquisition, compensation parameters were manually verified in FlowJo, and FMO controls were employed to refine gating.

RAW264.7 macrophage culture and AlamarBlue cytotoxicity assay

Murine RAW264.7 macrophage (ATCC, LGC Prochem, Midx, UK) were cultured in DMEM (Thermo Fisher Scientific) supplemented 10% v/v fetal bovine serum (FBS, Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 5% CO₂. Cells were passaged at approximately 80% confluence, and passages 5-8 were used for experiments. TMAO (99% purity, Sigma-Aldrich, NC, USA) and TMA 40% solution (Sigma-Aldrich, Steinheim, Germany) were dissolved in water at concentrations of 500 mM and 200 mM, respectively, as stock solutions were stored at -20°C and diluted freshly with media prior to cell treatment.

Cytotoxicity was determined by incubating the cells with alamarBlue reagent (Invitrogen, Life technologies, Carlsbad, CA, USA) according to manufacturer's protocol. RAW264.7 cells were cultured at a density of 2×10^5 cells per well in a 24-well plate overnight and treated with either TMAO at final concentrations of 5, 20, 100 and 500 μM , or TMA at the final concentrations of 5, 20, 100 and 150 μM for 24 h and 48 h. Etoposide (Sigma-Aldrich) was used as a positive control at a final concentration of 125 nM. AlamarBlue was added at a final concentration of 10% for each well and incubated for 3 h at 37°C. Fluorescence was measured in a BMG FLUOstar Omega Plate Reader (BMG, Labtech, Ortenberg, Germany) at excitation 560 nm/emission 590 nm. Cell viability of the treated cells was presented as a percentage of the negative control cells. Each sample was tested with technical triplicates, and three independent biological replicates were assessed.

Measurement of TNF- α in RAW264.7 cell culture supernatants

RAW264.7 cells were treated with TMAO (50 μM) and TMA (150 μM) for 24 h prior to lipopolysaccharide (LPS, Escherichia coli O111:B4) (10 ng/ml) treatment for 16 h. The protein levels of TNF- α in cell culture medium were determined by enzyme-linked immunosorbent assay (ELISA) kit against mouse TNF- α (R&D systems, Minneapolis, MN, USA, DY410) according to the manufacturer's instructions. Each sample was tested with duplicates, and three independent biological replicates were assessed.

HCT116 cell culture and cytotoxicity assay

Human colonic adenocarcinoma cell line HCT116 cells (ATCC, LGC Prochem, Midx, UK) were cultured in RPMI 1640 medium (Thermo Fisher Scientific) with 10% FBS at 37°C in a humidified atmosphere with 5% CO_2 . Cells at passages between 5-8 were used for experiments. For cytotoxicity measurement, HCT116 cells were seeded at a density of 3×10^4 per well in a 24-well plate for overnight and treated with TMAO at final concentrations of 5, 20, 100 and 500 μM for 24 h or 48h. Cell viability was assessed using the AlamarBlue assay as described above.

Proliferation and genotoxicity of HCT116 cells treated with TMAO

The effects of TMAO on HCT116 cell proliferation and DNA damage were assessed by flow cytometric analysis of Ki-67 (Invitrogen, Carlsbad, CA, USA) and γ H2AX (BioLegend, San Diego, CA, USA) staining, respectively, followed by flow cytometry analysis. HCT116 cells were cultured at a density of 4×10^5 cells per well in a 6-well plate for overnight. Cells were treated with TMAO (50 μ M) for 24h. Etoposide was used as a positive control at a final concentration of 125 nM and treated for 6 h as we previously observed a higher number of γ H2AX positive cells at this time point compared with 24 h. The cells were collected using enzyme-free cell dissociation buffer (GIBCO, Grand Island, NY, USA), and subsequently fixed and permeabilized. The fixed cells were stained with Ki-67 and γ H2AX antibodies and analyzed on a FACSymphony™ A3 flow cytometer (BD Bioscience, USA). The data were analyzed using FlowJo v10.8 (BD Bioscience, USA). Three independent biological replicates were performed.

Statistical Analysis

All experiments in the cellular study were performed with three independent biological replicates. Data are expressed as the mean $\pm$ SEM. Group differences were assessed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test for comparisons among multiple groups. For circulation TMAO and TMA concentrations in patients, data normal distribution was tested using Shapiro-Wilk test. Data with normal distribution was analyzed using paired t test or unpaired t test, and data that did not pass normality test were analyzed using Wilcoxon matched pairs signed rank test. In mouse experiments, the data were analyzed using either Kruskal-Wallis test or one-way ANOVA test. GraphPad Prism 9 software (CA, USA) was used for statistical analysis. $p < 0.05$ was considered statistically significant.

Declarations

MG is employed with the European Food Safety Authority (EFSA) in the Nutrition and Food Innovation Unit that provides scientific and administrative support to the Panel on Nutrition, Novel Foods, and Food Allergens, in the area of Novel Foods. However, the present work is published under the sole responsibility of the authors and may not be considered as an EFSA scientific output.

The positions and opinions presented in this article are those of the authors alone and do not necessarily represent the views or scientific work of EFSA.

Ethics approval and consent to participate

Serum samples were collected from 16 patients recruited from Emory University, USA (ethics reference: Institutional Review Board (IRB) #333-2002). The animal study was carried out under the Animals (Scientific Procedures) Act 1986 and received ethical approval (Project License: 9718F9C8) from the UK Home Office.

Consent for publication

Not applicable.

Competing interests

J.R.M. has received fees for consultation from Cultech Ltd. and Enterobiotix Ltd. The other authors declare no competing interests.

Authors' contributions

Yang Bi: Formal analysis, investigation, data curation, writing - original draft, writing - review & editing, visualization, project administration. **Maria Glymenaki:** Methodology, investigation, resources, writing - review & editing, visualization. **Maria A. Valdivia-Garcia:** Methodology, formal analysis, writing - review & editing, visualization. **Mu Wang:** Formal analysis, investigation, writing - review & editing, visualization. **Kai Yee Eng:** Investigation, writing - review & editing. **Edward Lin:** Resources, writing - review & editing. **Nana Gletsu-Miller:** Resources, writing - review & editing. **Julian R. Marchesi:** Resources, writing - review & editing, supervision. **Mona El-Bahrawy:** Investigation, writing - review & editing. **Nadia Guerra:** Methodology, writing - review & editing, supervision. **Jia V. Li:** Conceptualization, investigation, writing - original draft, writing - review & editing, supervision, project administration, funding acquisition.

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Source data

NMR spectral data are available at the MetaboLights data repository (Study number: MTBLS13866).

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Figure legend

Figure 1. Circulating methylamine concentrations in patients before and after bariatric surgery. (a) Circulating TMAO and **(b)** TMA concentrations in patients undergoing Roux-en-Y gastric bypass (RYGB) or laparoscopic gastric banding (LGB). Normality was assessed using the

Shapiro-Wilk test. Pre-operative versus 6-month values were compared using a paired t test for the LGB group and a Wilcoxon matched-pairs signed-rank test for the RYGB group. At 6 months, LGB and RYGB groups were compared using an unpaired t test. **(c)** Circulating TMAO concentrations in RYGB patients stratified by type 2 diabetes mellitus (T2DM) status. Pre-operative versus 6-month values were compared using a Wilcoxon matched-pairs signed-rank test in the non-T2DM group and a paired t test in the T2DM group. **(d)** Circulating TMA concentrations in RYGB patients, compared between pre-operative and 6-month time points using a paired t test. * $p < 0.05$, ** $p < 0.01$. Data are presented in mean $\pm$ SEM.

Figure 2. Baseline phenotypic characteristics of *FabplCre;Apc^{15lox/+}* mice. **(a)** Total intestinal length, **(b)** colon length, **(c)** spleen weight, **(d)** total tumor number, **(e)** number of colonic tumors, and **(f)** number of ileal tumors in female and male mice. **(g)** Kaplan–Meier survival curves for female and male mice ($p = 0.0032$, log-rank test). Distribution of tumor sizes in the **(h)** colon and **(i)** ileum, categorized as ≤ 0.1 cm, 0.1–0.5 cm, or ≥ 0.5 cm. Data in (a–f) are presented as mean $\pm$ SD.

Figure 3. Phenotypic characterization of *FabplCre;Apc^{15lox/+}* mice with or without dietary TMAO supplementation. **(a)** Experimental design of TMAO supplementation experiment using *FabplCre;Apc^{15lox/+}* mice. A total of 23 males and 24 females in the control group; and 23 males and 24 females in TMAO group. T0, starting point at 7 weeks of age; T1, midpoint, 10 and 11 weeks of age; and T2, endpoint, 13 and 15 weeks of age for male and female mice, respectively. **(b)** Body weight changes between weeks 6 and 13 for males and weeks 6–15 for females. **(c)** Food intake. **(d)** Gut length and **(e)** Colon length at the end of the experiment. **(f)** Proportion of mice that developed colonic tumors mice following either control or TMAO diet; statistical significance was assessed using Fisher's exact test. * $p < 0.05$ **(g)** total tumor volume per mouse in each group. Significance was determined by one-way ANOVA test. * $p < 0.05$. **(h)** Histological grading of colonic tumors. Left, representative H&E-stained sections of colonic tumors illustrating the histopathological scoring system used in this study. Score I: tubular adenoma with low-grade dysplasia; Score II: tubular adenoma with high-grade dysplasia; Score III: well-differentiated adenocarcinoma with preserved

glandular architecture; Score IV: moderately differentiated adenocarcinoma with increased architectural distortion and invasive features. Scale bars, 200 μ m. Right, doughnut plots showing scores of tumor grading based on the histological H&E staining for the colonic tissues and tumors from each group (5 males and 5 females in the control group; 6 males and 6 females in TMAO group). (i) Hematocrit values at T1 and T2 in controls and TMAO-supplemented mice. Data are shown as median and interquartile range as error bars. The slopes from T1 to T2 for all groups are statistically significant (Control males $p < 0.0001$; TMAO males $p = 0.0003$; Control females $p = 0.0001$; TMAO females $p < 0.0001$ by simple linear regression). At T2, males control vs TMAO, $p = 0.09$; females control vs TMAO, $p = 0.45$ by Dunn's multiple comparisons test.

Figure 4. Metabolic changes following dietary TMAO supplementation in male and female *Fabp1Cre;Apc^{15lox/+}* mice (a) TMAO and (b) TMA levels in jejunal, ileal, and colonic luminal contents. Urinary (c) TMAO and (d) TMA concentrations and plasma (e) TMAO and (f) TMA concentrations at T0, T1, and T2. (g) Plasma-to-urinary TMAO ratios at each time point. (h, i) Spearman correlation between circulating TMAO concentrations at T1 and T2 and tumor volume in (h) male and (i) female mice. Data are shown for control males (blue circles), TMAO-supplemented males (red circles), control females (blue triangles), and TMAO-supplemented females (red triangles). Statistical significance was assessed using the Kruskal-Wallis test (b, c) or one-way ANOVA (a, d–f). Comparisons in (g) were performed using Student's t test. * and # indicate significant differences between treatment groups of the same sex, and between males and females subjected to the same treatment, respectively. */# $p < 0.05$, **/## $p < 0.01$, ***/### $p < 0.001$ and ****/#### $p < 0.0001$. Data are presented in mean $\pm$ SEM.

Figure 5. Principal component analysis (PCA) of ¹H NMR metabolic profiles from urine, plasma, and intestinal luminal contents of *Fabp1Cre;Apc^{15lox/+}* following dietary TMAO supplementation. PCA scores plots are shown for (a) All urine samples colored by sex. Percentages of variation (R^2X) explained by the first and the second principal components (PC1 and PC2) are 22.10% and 9.73%, respectively. (b) All urine samples colored by sex, time point, and treatment; R^2X values of PC1 and PC2 are 22.10% and 9.73%, respectively. (c–d) Urine samples

from male and female mice, respectively, colored by timepoints and treatments; R^2X values of PC1 and PC2 are 23.90% and 11.70% in (c), and 13.10% and 7.82% in (d). **(e)** PCA scores plots from all plasma profiles; R^2X values of PC1 and PC2 are 13.00% and 4.53%, respectively. **(f-g)** Plasma samples from male and female mice, respectively; R^2X values of PC1 and PC2 are 14.10% and 7.30% in (f) and 11.80% and 5.82% in (g). PCA scores plots are shown for **(h)** all intestinal regions colored by anatomical locations and for **(i)** jejunal, **(j)** ileal, and **(k)** colonic contents colored by treatments. R^2X values of PC1 and PC2 are 34.80% and 10.60% in (h), 20.40% and 17.50% in (i), 20.30% and 19.60% in (j), and 15.10% and 8.45% in (k), respectively. Data were mean-centered and unit variance-scaled prior to PCA.

Figure 6. Immune profiles of colonic intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from *Fabp1Cre;Apc^{15lox+}* mice fed with normal chow (controls, Ctrl) or normal chow supplemented with TMAO. (a) Percentages of CD45⁺ cells in the colonic IEL and LPL. Statistical significance was assessed using the Kruskal-Wallis test. **(b)** Composition of immune cell subsets in IEL and LPL from control and TMAO-supplemented groups. Cells were gated on non-autofluorescence lymphocytes with subsequent gating as follows: CD4⁺ T cells: CD3⁺CD4⁺; CD8⁺Tcells: CD3⁺CD8⁺; $\gamma\delta$ T cells: CD3⁺TCR $\gamma\delta$ ⁺; Myeloid cells: CD3⁻CD19⁻NK1.1⁻CD11b⁺ and NK cells: CD3⁻NK1.1⁺. **(c)** Fold changes (ratios) in immune cell subset frequencies in TMAO-supplemented mice relative to controls. **(d)** Ratios of CD8⁺ T cells to Foxp3⁺ cells in colonic IEL and LPL compartments. **(e)** Percentages of TNF α -expressing cells within each subset (CD8⁺, CD4⁺, $\gamma\delta$ T and NK cells) in the IEL and representative gating strategies in flow cytometry analysis. **(f)** Percentages of GrB⁺-expressing cells within total $\gamma\delta$ T cells in IEL and representative gating strategies in flow cytometry analysis. **(g)** Spearman's correlation analysis between circulating TMAO levels at T1 and TNF- α expressing cell frequencies in the total non-autofluorescent CD45⁺ cells. Significance was determined by Kruskal-Wallis test. * $p < 0.05$, ** $p < 0.01$. Data are presented in mean $\pm$ SEM, $n=6$ per group per sex.

Figure 7. TNF- α expression in colonic intraepithelial (IEL) and lamina propria lymphocytes (LPL) populations following dietary TMAO supplementation. (a, b) Frequencies of TNF- α ⁺ cells

within immune cell subsets in colonic IELs from *Fabp1Cre;Apc^{15lox+}* mice fed with normal chow (controls, Ctrl) or normal chow supplemented with TMAO. **(c–e)** Frequencies of TNF- α cells within immune cell subsets in colonic LPLs from controls and TMAO-supplemented mice. Statistical significance was assessed using the Kruskal-Wallis test. Data are presented in mean $\pm$ SEM, n=6 per group per sex.

Figure 8. Immune profiles of duodenum-jejunum and ileal intraepithelial (IEL) and lamina propria lymphocytes (LPL) following dietary TMAO supplementation. (a–f) Immune profiles of duodenum-jejunum IELs and LPLs from control and TMAO-supplemented *Fabp1Cre;Apc^{15lox+}* mice. **(a, d)** Frequencies of CD45+ cells in IEL and LPL compartments. **(b, e)** Composition of immune cell subsets in IELs and LPLs. Immune populations were identified following exclusion of auto-fluorescent cells and defined as follows: CD4+ T cells, CD3+CD4+; CD8+ T cells, CD3+CD8+; $\gamma\delta$ T cells, CD3+TCR $\gamma\delta$ +; myeloid cells, CD3–CD19–NK1.1–CD11b+; and NK cells, CD3–NK1.1+. **(c, f)** Fold changes in immune cell subset frequencies in TMAO-supplemented mice relative to controls. **(g–i)** Immune profiles of ileal IELs and LPLs. **(g, j)** Frequencies of CD45+ cells; **(h, k)** composition of immune cell subsets; and **(i, l)** fold changes in immune cell subset frequencies in TMAO-supplemented mice relative to controls. Statistical significance was assessed using the Kruskal-Wallis test. Data are presented as mean $\pm$ SEM; n = 6 mice per treatment group per sex.

Figure 9. Effects of TMAO and TMA on RAW264.7 macrophage viability and TNF- α production. (a–d) Viability of RAW264.7 macrophages following treatment with TMAO of 5, 20, 100 and 500 μ M for 24 h **(a)** and 48 h **(b)**, or TMA at concentrations of 5, 20, 100 and 150 μ M for 24 h **(c)** and 48 h **(d)**, assessed using the AlamarBlue assay. Cell viability is expressed relative to untreated controls. **(e)** TNF- α concentrations in culture supernatants from RAW264.7 cells pretreated with TMAO (50 μ M) or TMA (150 μ M) for 24 h, followed by LPS stimulation (10 ng/mL) for 16 h. Statistical significance was assessed using one-way ANOVA. One-way ANOVA was used and ***p < 0.001. ns, no significance. Data are presented in mean $\pm$ SEM, three independent biological replicates per treatment group.

767 **Figure 10. Cytotoxic and genotoxicity effects of TMAO on human colonic HCT116 cells.** Cells
768 were treated with TMAO at concentrations of 5, 20, 100 and 500 μ M for **(a)** 24 h and **(b)** 48 h. Cell
769 viability following TMAO treatment was determined by AlamarBlue assay. Cell viability is expressed
770 relative to untreated controls. **(c)** Expression levels of Ki-67 and γ H2AX staining in HCT116 cells
771 following 50 μ M TMAO treatment for 24 h, assessed by flow cytometry. Data is expressed relative
772 to untreated controls. Etoposide (ETO) served as positive controls. * $p < 0.05$; ns, not significant.
773 Data are presented as mean $\pm$ SEM from three independent biological replicates.

774

Tables

Table 1. Demographic and clinical characteristics of patients who underwent bariatric surgery at baseline (pre-op) and 6 months post-op (6 mo).

	LGB			RYGB		
	pre-op	6 mo	p	pre-op	6 mo	p
Number of patients (glycemia)	5 (1)			11 (6)		
Race (Black:White)	4:1			4:7		
Age (years)	31 ± 9.09			41 ± 8.21		
VAT (cm ³)	2696 ± 1859.95	2272 ± 1319.84	ns	6022 ± 2209.08	2096 ± 1378.57	0.001
SAT (cm ³)	12674 ± 2557.7	10806 ± 3427.74	ns	16601 ± 4408.35	9564 ± 3258.91	0.001
CRP	0.79 ± 0.33	0.2 ± 0.43	ns	1.28 ± 1.15	0.41 ± 0.48	0.004
FAT (kg)	53.3 ± 14.29	43.5 ± 15.21	ns	70 ± 12.87	40.2 ± 13.41	0.001
WC (cm)	114.2 ± 11.28	99.2 ± 10.47	ns	126.8 ± 14.36	102 ± 13.49	0.001
GLC (mM)	4.28 ± 0.48	4.83 ± 1.76	ns	6.06 ± 1.75	4.2 ± 0.59	0.001
INS (uIU/mL)	15.6 ± 18.58	6.5 ± 3.22	ns	11.4 ± 9.37	3.1 ± 1.41	0.001
HOMA	2.84 ± 4.6	1.61 ± 0.52	ns	2.63 ± 4.28	0.57 ± 0.33	0.001
BMI (kg/m ²)	39.59 ± 5.72	34.86 ± 6.43	ns	45.27 ± 3.43	34.26 ± 4.79	0.001
SBP (mmHg)	115.5 ± 15.78	128 ± 10.52	ns	137 ± 17.42	119.4 ± 16.33	0.011
DBP (mmHg)	65 ± 10.21	76.7 ± 9.74	ns	72.3 ± 11.88	71 ± 11.06	ns
REE (kcal)	2327 ± 241.64	1720 ± 260.52	ns	2051 ± 415.81	1647 ± 284.13	0.002

Key: BMI, body mass index; CRP, high sensitivity C-reactive protein; DBP, diastolic blood pressure; FAT, body fat mass by air displacement plethysmography; GLC, fasting glucose; HOMA, homeostasis model assessment; INS, fasting insulin; LGB, Laparoscopic Adjustable Gastric Banding; REE, resting energy expenditure by metabolic cart; RYGB, Roux-en-Y gastric bypass; SAT, abdominal subcutaneous adiposity volume by computed tomography; SBP, systolic blood pressure; VAT, abdominal visceral adiposity volume by computed tomography; WC, waist circumference. Data are shown as median ± standard deviation; p values derived from Wilcoxon matched-pairs signed rank test (two-tailed); ns, non-statistically significant (p>0.05).

791 **Table 2.** A list of antibodies used in flow cytometry.

Antigen	Clone	Concentration ($\mu\text{g/mL}$)	Fluorophore	Supplier
CD8	53-6.7	0.5	BUV395	BD Biosciences
CD4	GK1.5	0.5	BUV496	BD Biosciences
Ly6G	1A8	0.5	BUV661	BD Biosciences
CD3	145-2C11	1	BUV737	BD Biosciences
F4/80	BM8	0.5	Brilliant Violet 421	Biolegend
IFN γ	XMG1.2	2	eFluor 450	eBioscience
CD11b	M1/70.15.11.5	n/a (dilution 1:800)	VioGreen	Miltenyi Biotec
Ly6C	HK1.4	0.025	Brilliant Violet 605	Biolegend
NK1.1	PK136	1	Brilliant Violet 650	Biolegend
T-bet	4B10	4	Brilliant Violet 711	Biolegend
B220	RA3-6B2	0.25	Brilliant Violet 785	Biolegend
CD200R	REA850	n/a (dilution 1:100)	Vio Bright B515	Miltenyi Biotec
CD127	A7R34	5	FITC	eBioscience
CD107a (LAMP-1)	1D4B	5	Biotin	Biolegend
CD206 (MMR)	C068C2	2	PerCP/Cyanine5.5	Biolegend
CD279 (PD-1)	J43	1	PerCP-eFluor 710	eBioscience
CD314 (NKG2D)	CX5	2	PE	eBioscience
ROR γ t	B2D	2	PE-eFluor 610	eBioscience
$\gamma\delta$ TCR	eBioGL3 (GL-3, GL3)	0.5	PE-Cyanine5	eBioscience
IL-17	TC11-18H10.1	2	PE/Cyanine7	Biolegend
Foxp3	FJK-16s	1	APC	eBioscience
GrB	GB11	2	Alexa Fluor 647	BD Biosciences
CD45	30-F11	0.5	Alexa Fluor 700	Biolegend
TNF- α	MP6-XT22	2	APC/Cyanine7	Biolegend

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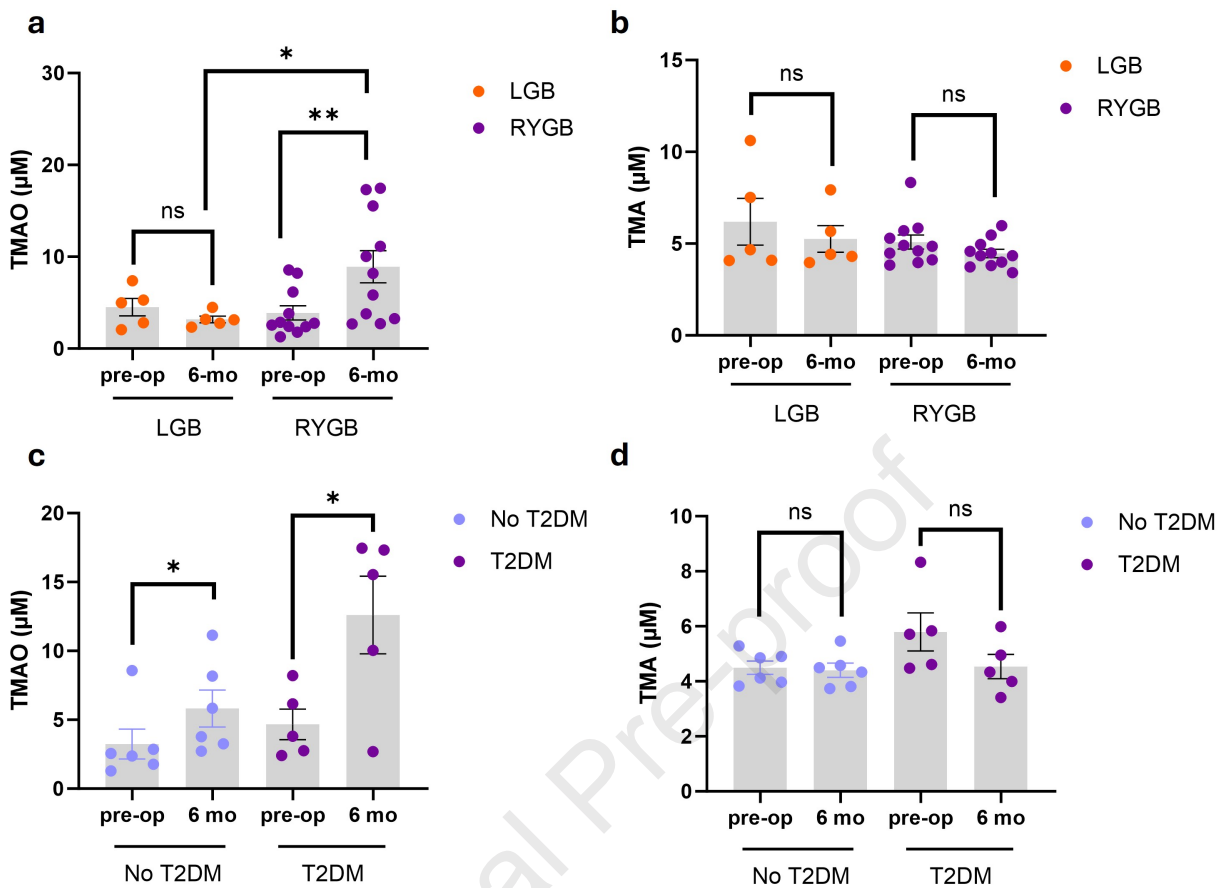
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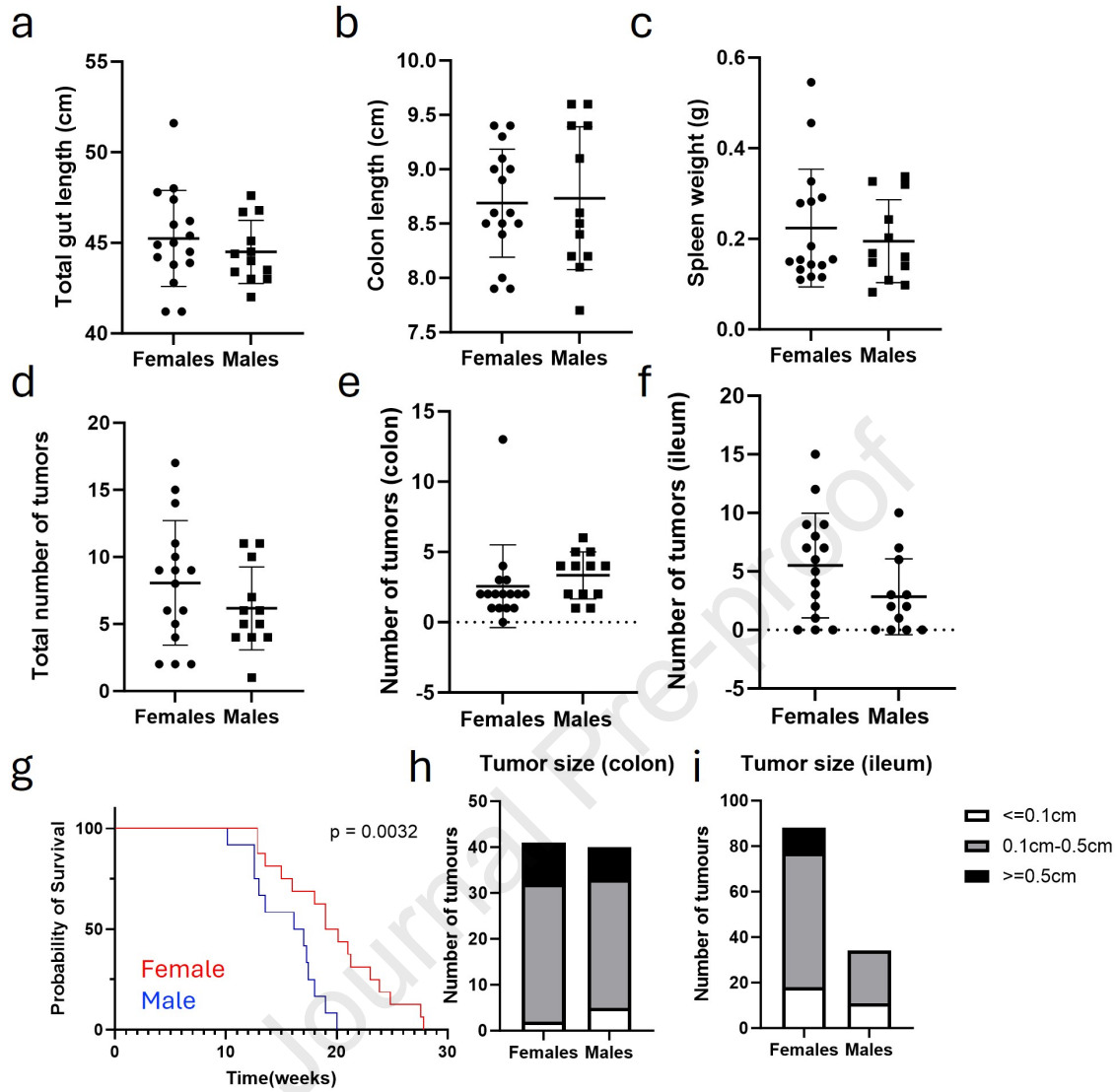
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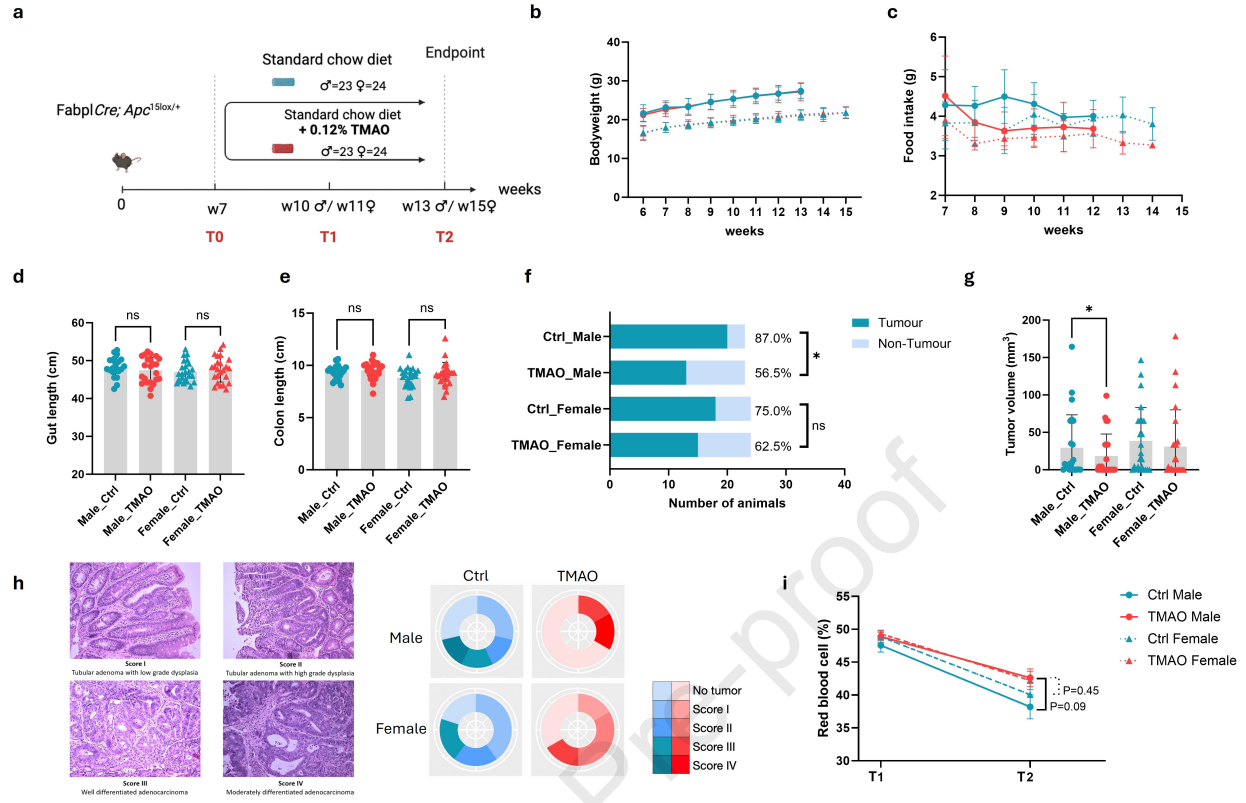
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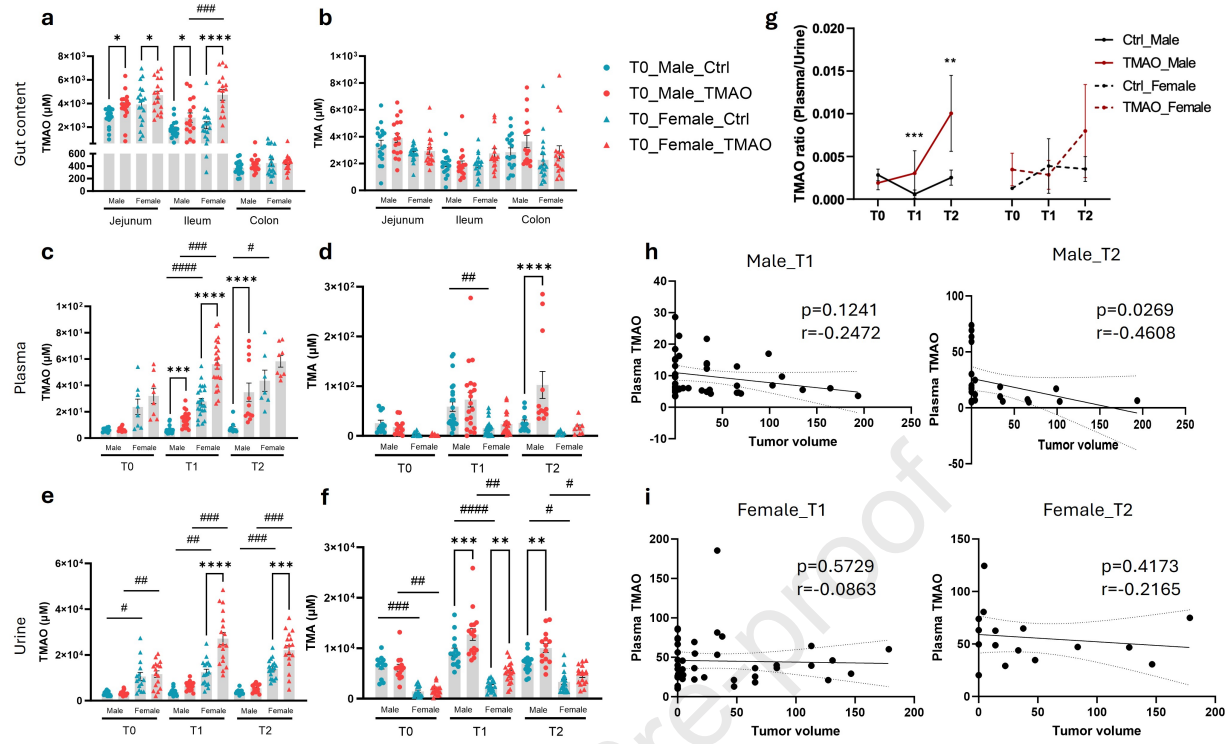
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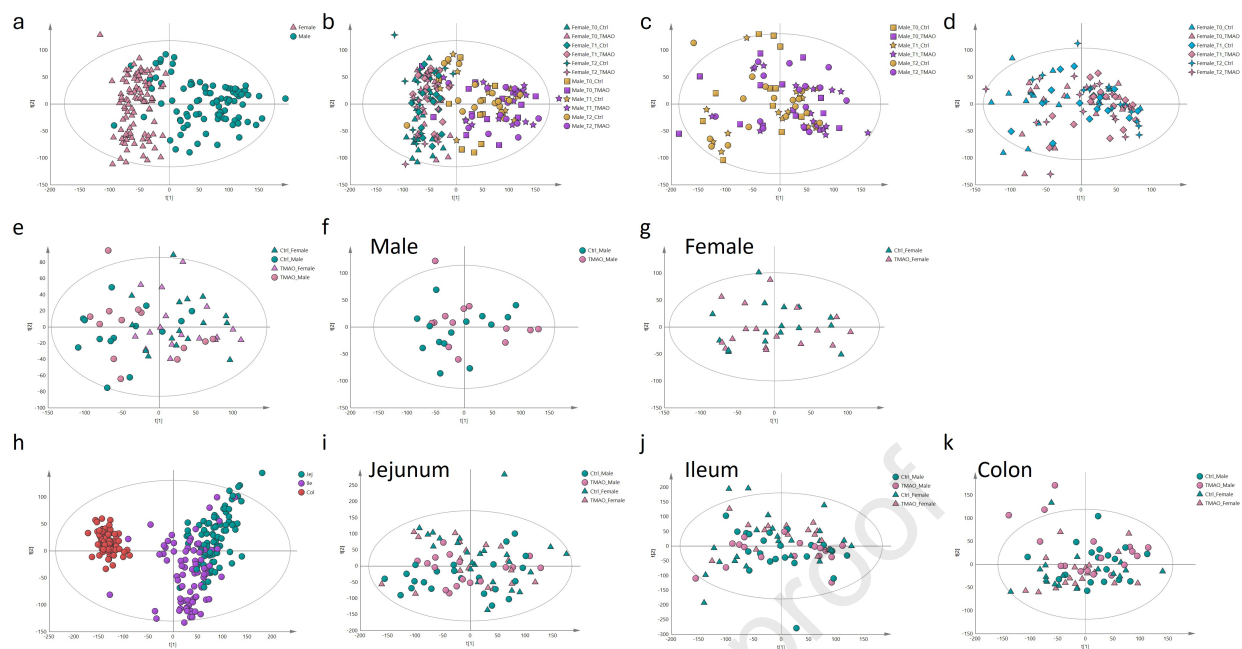
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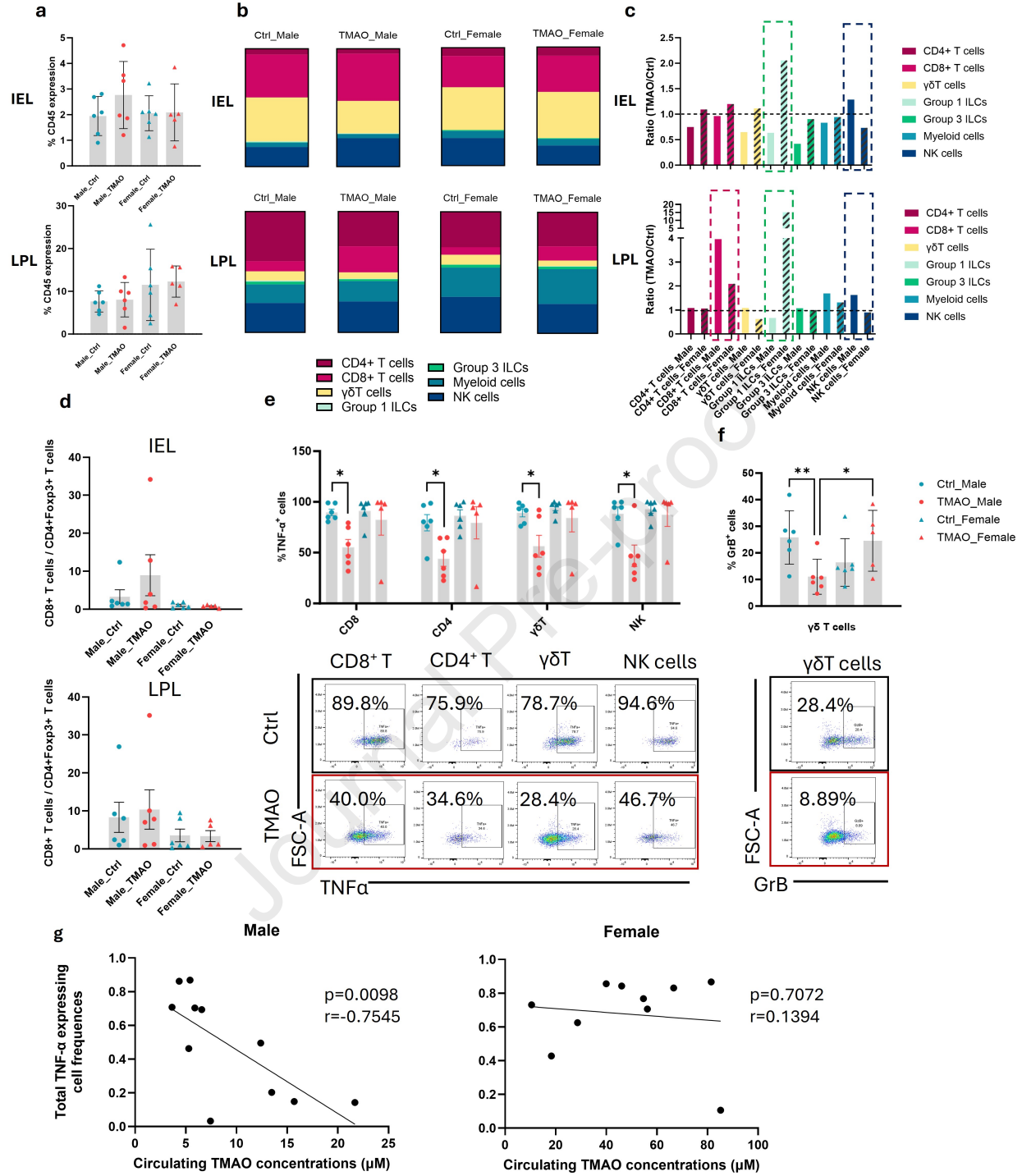


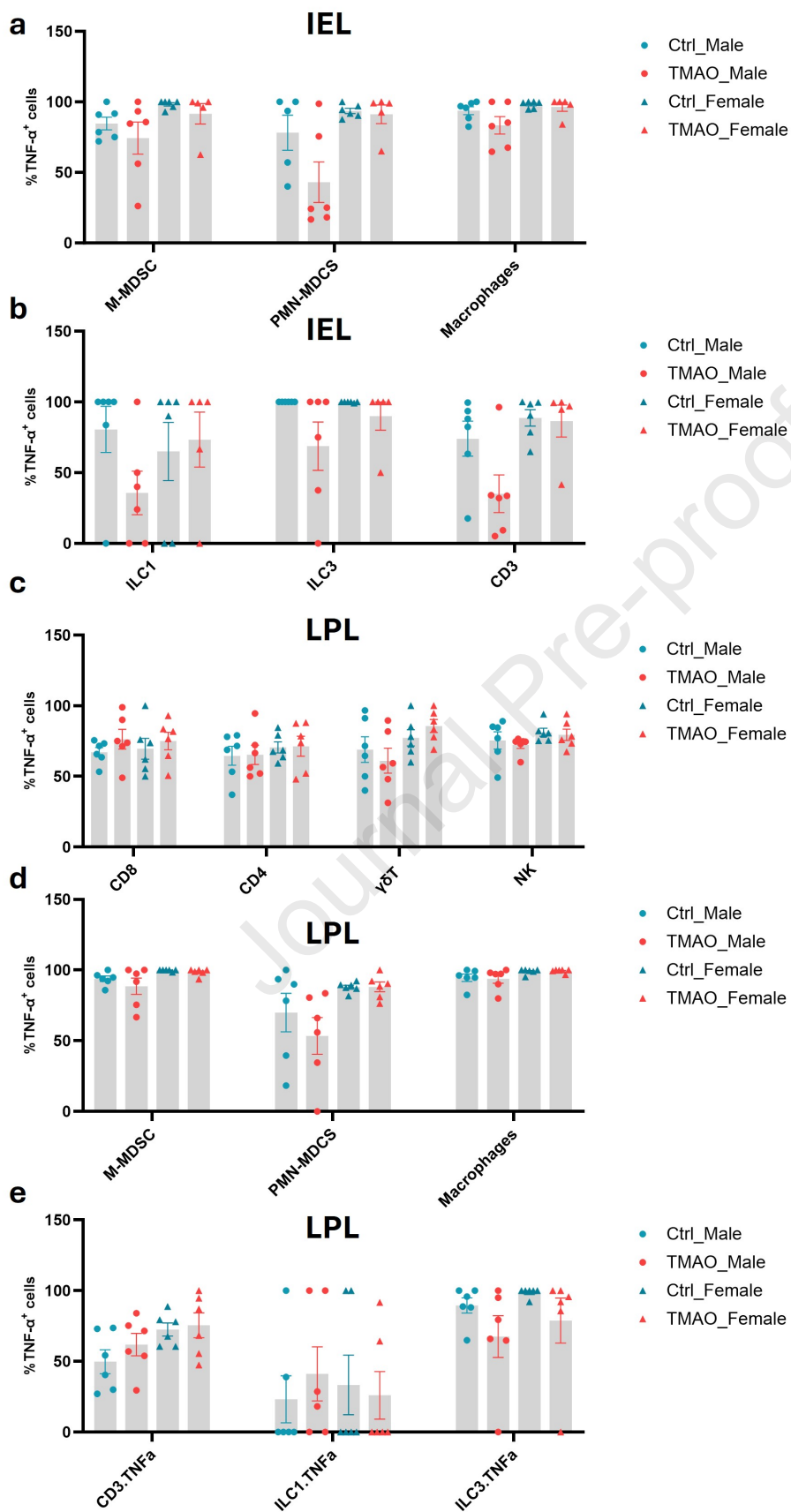


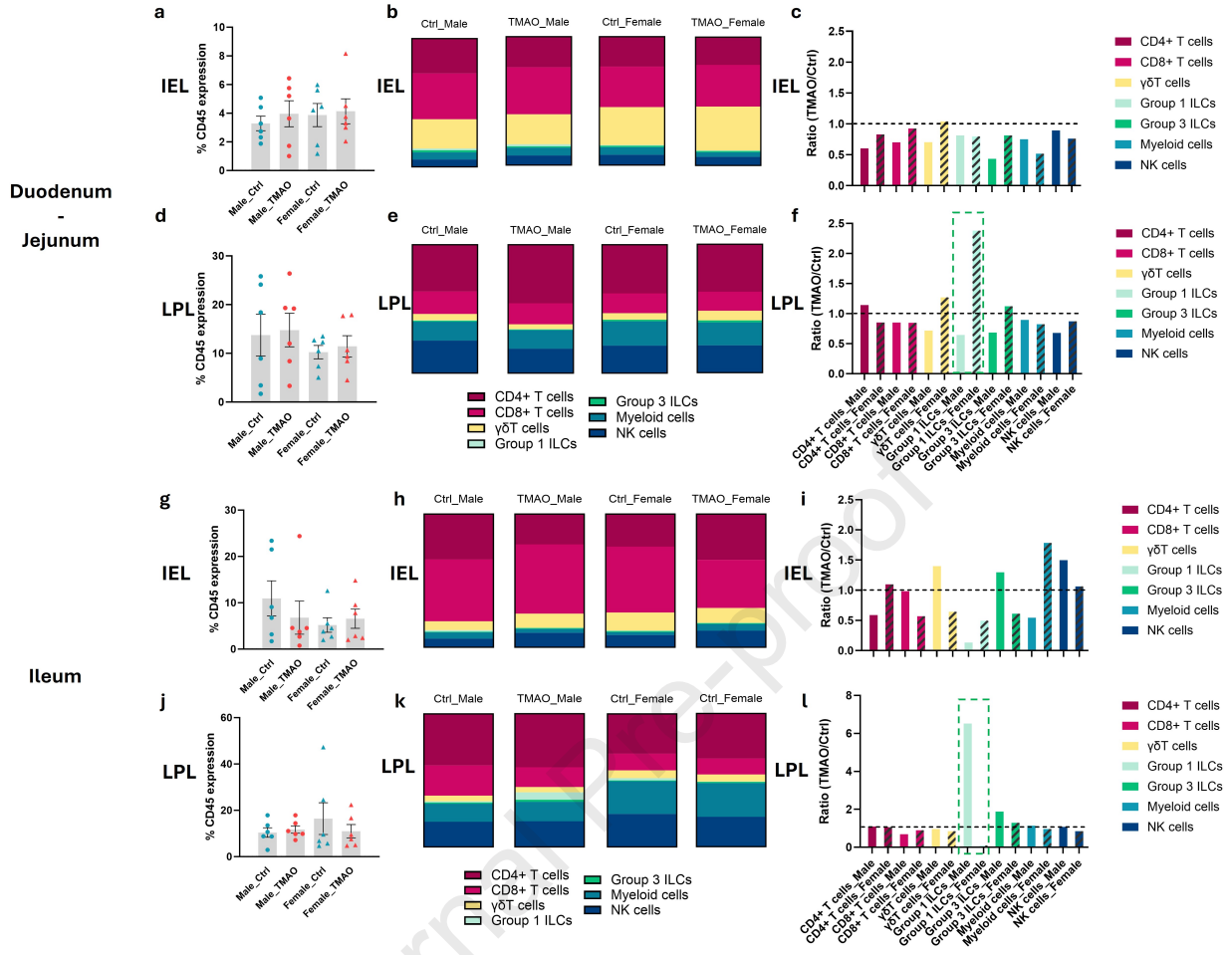


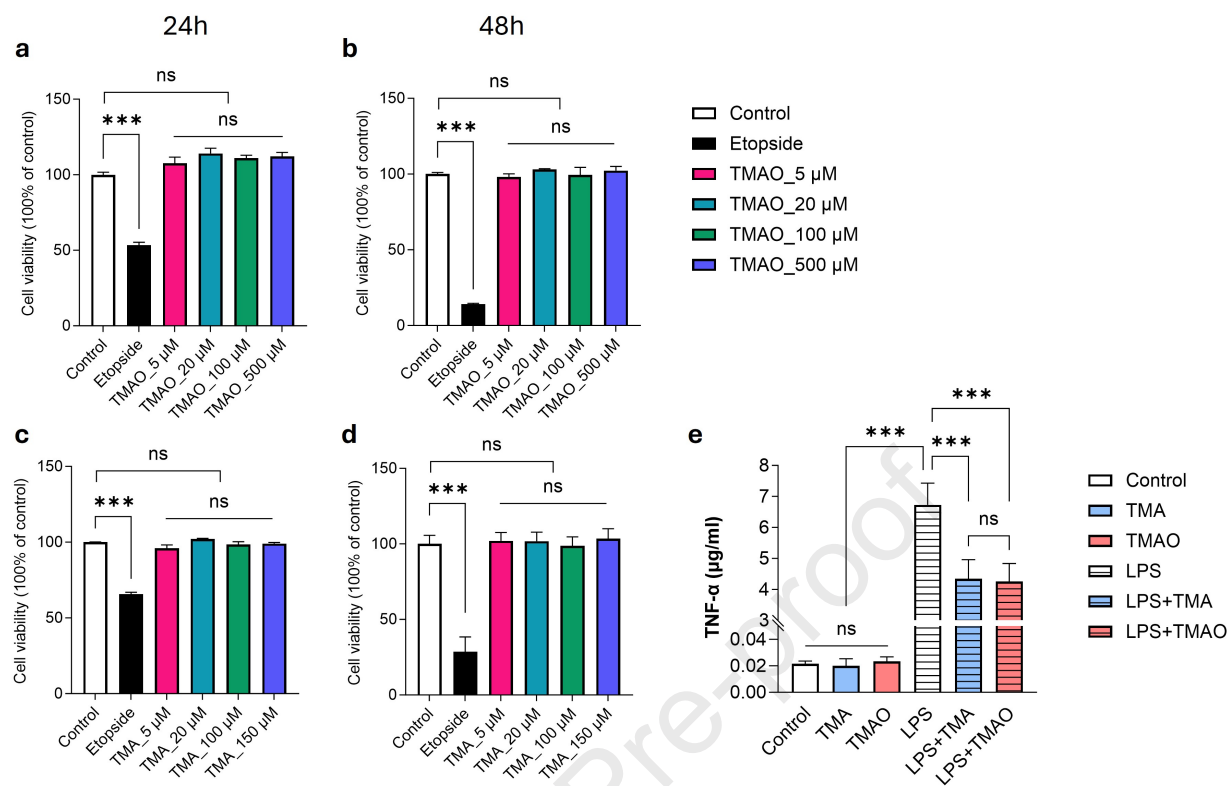


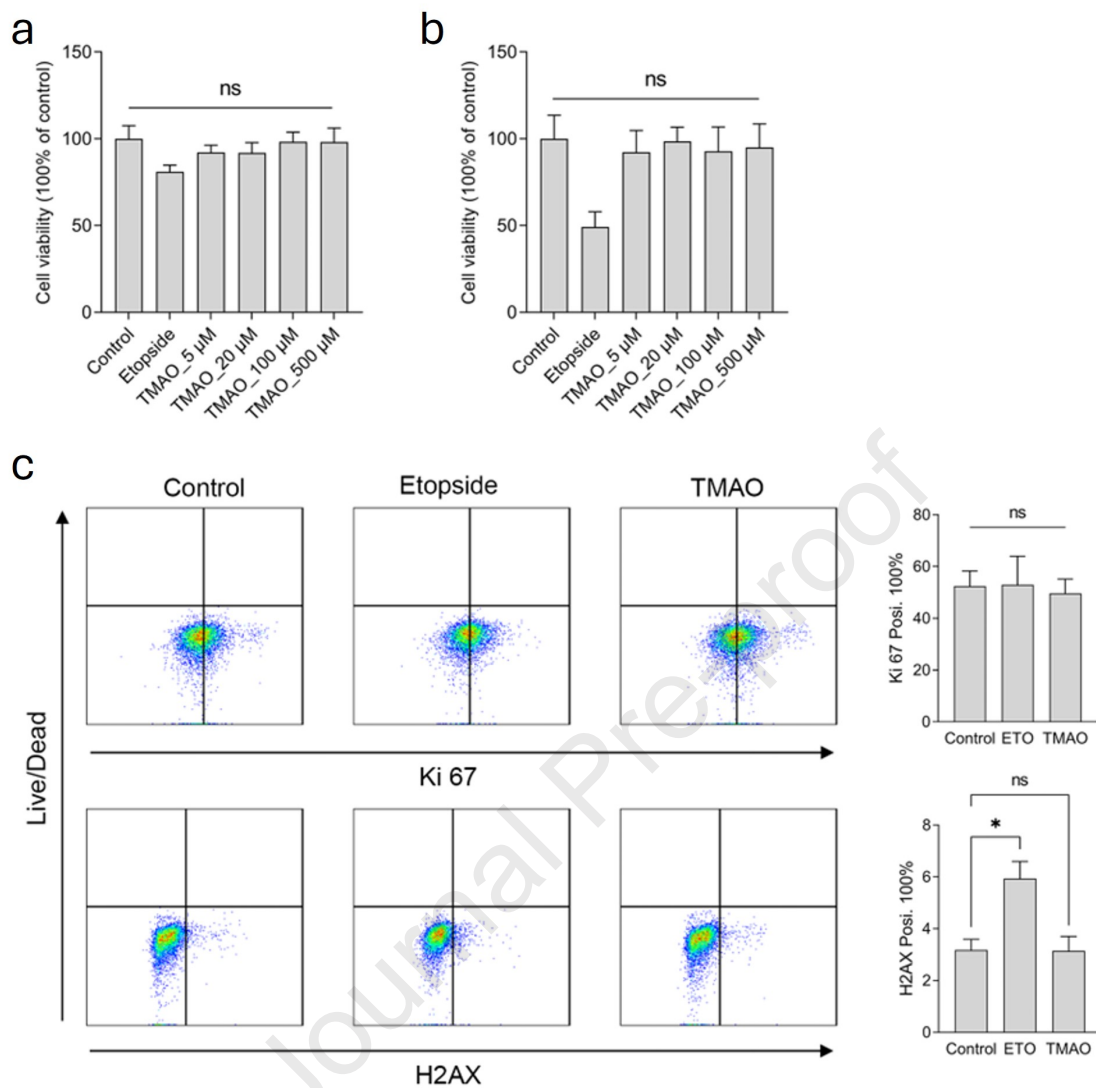












What You Need to Know

Background

Trimethylamine *N*-oxide (TMAO) increases markedly after Roux-en-Y gastric bypass (RYGB). RYGB has been associated with an elevated risk of colorectal cancer (CRC), although evidence remains mixed.

Impact

Dietary TMAO reduced colonic tumor burden and increased systemic TMAO retention in male mice, suggesting a sex-dependent protective effect potentially mediated by reduced TNF- α -expressing colonic intraepithelial lymphocytes.

Future Directions

Elucidating TMAO-mediated modulation of TNF- α signalling in human populations, together with mechanisms underlying sex-specific TMAO retention, is essential for informing targeted dietary interventions aimed at reducing colorectal cancer risk.