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Dietary manipulation of oncogenic microRNA expression in human rectal mucosa: a randomised trial

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ABSTRACT

High red meat intake is associated with increased colorectal cancer (CRC) risk, while resistant starch is probably protective. Resistant starch fermentation produces butyrate, which can alter microRNA (miRNA) levels in CRC cells \textit{in vitro}; effects of red meat and resistant starch on miRNA expression \textit{in vivo} were unknown. This study examined whether a high red meat (HRM) diet altered miRNA expression in rectal mucosa tissue of healthy volunteers, and if supplementation with butyrylated resistant starch (HRM+HAMSB) modified this response. In a randomised cross-over design, twenty-three volunteers undertook four 4-week dietary interventions; an HRM diet (300 g/day lean red meat) and an HRM+HAMSB diet (HRM with 40 g/day butyrylated high amylose maize starch), preceded by an entry diet and separated by a washout. Faecal butyrate increased with the HRM+HAMSB diet. Levels of oncogenic mature miRNAs, including miR-17-92 cluster miRNAs and miR-21, increased in the rectal mucosa with the HRM diet, while the HRM+HAMSB diet restored miR-17-92 miRNAs, but not miR-21, to baseline levels. Elevated miR-17-92 and miR-21 in the HRM diet corresponded with increased cell proliferation, and a decrease in miR-17-92 target gene transcript levels, including \textit{CDKN1A}. The oncogenic miR-17-92 cluster is differentially regulated by dietary factors that increase or decrease risk for CRC, and this may explain, at least in part, the respective risk profiles of high red meat and resistant starch. These findings support increased resistant starch consumption as a means of reducing risk associated with an HRM diet.
INTRODUCTION

The majority of colorectal cancers (CRCs) occur sporadically, with development influenced by environmental and lifestyle factors, including diet (1). Systematic reviews of cohort and case-control studies have found high red meat or processed meat intake to be a convincing risk factor (1, 2), with intake of more than 500 g of cooked red meat per week significantly increasing CRC risk (1). Plausible mechanisms include inducing DNA strand breaks and enhancing pro-mutagenic DNA adduct formation (3, 4). High red meat consumption has also been linked to gut microbiome changes and inflammation (5, 6). In contrast, dietary fibre probably protects against CRC, with systematic review evidence identifying a dose-response relationship, with 10% decreased risk per 10 g fibre intake per day (1). Interventional studies provide less conclusive evidence, and longer-term trials and higher fibre levels may be needed to reproduce effects from observational studies (7).

One protective mechanism for fibre is the production of fermentation products, particularly the short-chain fatty acid (SCFA) butyrate (1). Butyrate is a histone deacetylase inhibitor, with anti-tumorigenic effects (8-12). Aberrant microRNA (miRNA) expression contributes to CRC development (13-15), with miRNAs such as miR-21 and the miR-17-92 cluster of miRNAs often increased in CRCs and possessing oncogenic properties (16, 17). We have shown that butyrate can modulate miRNA expression in CRC cells in vitro (18). The miR-17-92 cluster, comprising miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a, was significantly decreased with butyrate. This decrease may be partially responsible for the anti-proliferative effects of butyrate, with addition of miR-17-92 mimics reversing this and increasing proliferation; miR-19a and b in particular were key promoters of proliferation.
Through epigenetic mechanisms, butyrate may be able to reverse the miRNA dysregulation observed in CRC (18).

Higher colonic butyrate levels can be achieved with resistant starch supplementation compared with other fibre sources (19). Resistant starch can also be acetylated with butyrate; butyrylated high amylose maize starch (HAMSB) can deliver esterified butyrate to the human colon, leading to increased faecal butyrate compared with standard high amylose maize starch (P < 0.0001) (20, 21). In rodents, resistant starch supplementation to a high red meat diet increased colonic butyrate, altered gut microbiota, decreased inflammation, and attenuated red meat-induced DNA damage (3, 5, 22). HAMSB was more effective than standard amylose maize starch in lowering genetic damage (23). One human trial has suggested inconclusively that fibre may modify DNA adduct formation in the context of high red meat consumption (4); however, to date no other human trials have examined the combined effects of red meat and resistant starch. There has been no previous examination in vivo of the effects of these substances on miRNA expression in colorectal cells.

This human trial aimed to determine if consumption of a diet high in lean red meat altered miRNA expression in rectal mucosa tissue, and if supplementation with resistant starch could protect against this dysregulation by increasing butyrate levels in the colorectum. In a randomised cross-over design, markers of CRC risk were measured in healthy human volunteers who undertook two four-week intervention diets, a high red meat (HRM) diet, and a high red meat diet supplemented with butyrylated resistant starch (HRM+HAMSB) (StarPlus™, National Starch and Food Innovation, Bridgewater, NJ). It was hypothesised that regulation of miRNA expression may partially explain some of the chemo-protective effects of resistant starch and the increased CRC risk associated with high red meat intake.
MATERIALS AND METHODS

Subjects
The randomised, controlled cross-over trial was approved by the Flinders Clinical Research Ethics Committee and Clinical Drug Trials Committee, and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12609000306213). Sample size was based on the anticipated effect on a primary outcome (fermentation products). A group size of \( n = 20 \) gave 80% power to detect a 20% change with 95% probability. Twenty-five were recruited to allow for drop-outs. A computer-generated randomisation sequence was implemented by a trial nurse, to determine which intervention diet was received first. Due to the nature of the interventions, participants were not blinded. Healthy volunteers aged 50 – 75 years, with no active bowel disease, and able to provide informed consent were eligible for inclusion. Exclusion criteria included evidence of active bowel disease or malabsorption, intolerance to high-fibre foods, perceived contraindication to consumption of the high protein diet, previous bowel surgery (excluding polypectomy), ingestion of regular laxatives or probiotic complimentary medicines, and antibiotic therapy in the previous four weeks. Patients were recruited by advertisement or invitation from their physician at the Flinders Medical Centre gastroenterology outpatient clinics, and written informed consent was obtained. Participants could withdraw at any time.

Study design
Dietary interventions were explained during clinic visits. The study consisted of two intervention periods of four weeks each, preceded by a four-week run-in (entry) period and separated by a four-week washout period. Volunteers were randomised to an HRM diet (300 g raw weight of lean red meat per day) or HRM+HAMSB diet (300 g raw weight of lean red
meat per day with 40 g of butyrylated high amylose maize starch per day [StarPlus™, which is 50 – 60% resistant starch](24)). Volunteers received the alternative diet for the second intervention. Meat was supplied in frozen packs of lean mince, beef strips, or lamb strips. HAMSB was supplied as pre-packed 20 g sachets, with two to be consumed daily by mixing into 250 mL reduced fat milk or orange juice. During the HRM diet, participants were also required to consume two serves of reduced fat milk or orange juice per day, to match the HRM+HAMSB diet. The level of red meat used is tolerated well, with studies often using 400 g of red meat per day (4). Intervention studies have shown that 40 g per day of butyrylated resistant starch significantly raises colonic butyrate concentrations (21).

Participants were to maintain their usual diet during the study but to avoid additional high-protein, fibre or probiotic supplements, and any medication that could interfere with bowel function. Participants were monitored by a trial nurse and dietitian, to ensure intervention guidelines were followed, and weight was kept stable.

**Sample collection and analysis**

The effects of the dietary interventions on colonic fermentation product formation and on epithelial consequences were examined as primary outcomes. SCFA were measured from faecal samples, and miRNA expression changes, target gene levels, and cell proliferation were measured from rectal mucosa samples. Faecal and rectal pinch biopsy specimens were obtained at the end of each four-week dietary period. Details of medical history and medications, weight, bowel health, and adverse events were collected throughout the study. Composition of the participants’ diets and compliance with the interventions was assessed using weighed food diaries, completed by participants for the last three days of each four-week dietary period. Food diaries were entered into Foodworks Professional 7 nutritional calculation software (Xyris Software, Kenmore Hills, QLD, Australia) by a dietitian, to
calculate energy and macronutrient intake based on Australian food composition tables and food manufacturers’ data.

Faecal collection was conducted by participants for the last 24 h of each dietary period. Samples were homogenized in three volumes of internal standard solution (1.68 mmol heptanoic acid/L) and centrifuged. The supernatant was vacuum distilled, and 0.2 µL of each distillate was loaded onto a Zebron ZB-FFAP gas chromatography (GC) column (Phenomenex, Torrance, CA) within an Agilent 6890N Network GC system (Agilent, Santa Clara, CA). Concentrations of acetate, butyrate, propionate, and total SCFAs were reported.

Participants undertook anal examination by an experienced gastroenterologist at the end of each dietary period, and four pinch rectal biopsies were taken with forceps through sigmoidoscopic examination performed without bowel preparation. Two biopsies of <0.5 cm in any dimension were stored in RNAlater (Ambion, Foster City, CA). Additional biopsies were formalin-fixed. Biopsies stored in RNAlater were used for quantitation of miRNA and target gene mRNA levels. Each biopsy was homogenised in 0.5 mL TRIzol Reagent (Ambion, Foster City, CA), and total RNA extracted according to the manufacturer’s instructions. RNA was quantified using a Nanodrop-8000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

miRNA expression analysis was conducted by relative quantitation real-time RT-PCR using TaqMan miRNA assays (Applied Biosystems, Foster City, CA). cDNA was synthesized from 20 ng total RNA using miRNA-specific primers, and real-time PCR was carried out using triplicate 10 µl reactions for each biological replicate including 1 µl of reverse transcription product, 0.5 µl miRNA-specific primer, and probe assay mix, and 1 × TaqMan Universal
PCR Master Mix No AmpErase UNG (Applied Biosystems) (assay IDs: miR-17: 000393, miR-18a: 002422, miR-19a: 000395, miR-20a: 000580, miR-19b: 000396, miR-92a: 000430, miR-16: 000391, miR-21: 000397). Results were normalized relative to the endogenous small nuclear RNA gene RNU6B (assay ID: 001093) using Qgene (25). For mRNA expression analysis, real-time RT-PCR was conducted using TaqMan Gene Expression assays (Applied Biosystems). cDNA was synthesized from 0.6 µg total RNA using M-MLV Reverse Transcriptase, RNase H minus (Promega, Madison, WI) and random hexamer primers. Real-time PCR was carried out using triplicate 10 µl reactions including 2 µl of RT product, 0.5 µl mRNA-specific Gene Expression assay mix, and 1× TaqMan Gene Expression master mix (Applied Biosystems) (assay IDs: CDKN1A: Hs00355782_m1, PTEN: Hs02621230_s1, BCL2L11: Hs00708019_s1). Results were normalized relative to endogenous controls ACTB (β-actin) (assay ID: Hs99999903_m1) and B2M (β-2-microglobulin) (assay ID: Hs00984230_m1) using QbasePlus (Biogazelle, Zwijnaarde, Belgium).

To assess the proliferative activity and distribution of proliferating cells in the colonic crypts, the proliferating cell nuclear antigen (PCNA) assay was performed using standard immunohistochemical procedures (11). Deparaffinised rectal biopsy sections were rehydrated in a graded series of ethanol from 100 to 50% and then distilled water. Primary mouse monoclonal antibody (PC10) (Santa Cruz Biotechnology, Santa Cruz, CA) was placed on slides (1:500 dilution) and incubated overnight, followed by incubations with a murine ultra streptavidin HRP detection kit (Covance Laboratories, Madison, WI). Visualisation was performed using 3,3’-diaminobenzidine (DAB) chromagen and substrate buffer (Covance Laboratories), with slides counterstained with haematoxylin. PCNA-positive cells were identified in 20 randomly chosen intact crypts by an independent observer who was blinded to the treatment groups.
**Statistical analysis**

Data were presented as mean ± standard error of the mean (SEM), with graphs prepared using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA). Statistical analyses were performed in IBM SPSS Statistics 22 (IBM SPSS Inc, Chicago, IL) using the repeated measures general linear model, with statistical significance for paired comparisons obtained using Sidak multiple comparisons test. For each variable, data were assessed for carry-over and period effects. For variables with no significant carry-over or period effects, data from both intervention periods were combined and the repeated measures analysis was used. Secondary analyses were performed for outcomes where there was a significant effect of treatment order, with the group that consumed the HRM diet first analysed independently from the group that consumed the HRM+HAMSB diet first. An adjusted P value < 0.05 was considered significant.
RESULTS

Demographic data and dietary intake
Recruitment commenced in July 2009, with participants followed-up for the four month duration of the interventions. Data collection was completed by September 2010. Twenty-five participants were randomly assigned, with 12 allocated to the HRM dietary intervention first, and 13 allocated to the HRM+HAMSB dietary intervention first (Figure 1). Two participants withdrew prior to commencing the interventions; one due to unrelated medical problems, and one due to intolerance of the first rectal biopsy. At study completion, 23 participants had received both intervention diets (17 males and 6 females, aged 50 – 75 years), and data from these participants were analysed. There were no major complications, and all 23 volunteers tolerated sample collection and the interventions, except one volunteer who usually had a vegetarian diet and found 300 g red meat per day difficult to tolerate. Approximately one third of volunteers reported increased flatulence on trial diets; it was unclear whether this could be linked to increased red meat or resistant starch intake. Participants maintained consistent body weight (mean of 80 kg after each diet; P > 0.05). Weighed food diaries showed that compared with the entry diet, protein intake was significantly increased in the HRM diet (P < 0.0001) and in the HRM+HAMSB diet (P = 0.001); intake was similar in the HRM and HRM+HAMSB diets (P = 0.33). Fibre and starch intake were decreased in the HRM diet compared with the entry (P < 0.0001 and P = 0.02 respectively) and HRM+HAMSB diets (P < 0.0001 and P = 0.02 respectively).

Faecal SCFA levels
There was a significant increase in acetate (P = 0.002), propionate (P = 0.0006), butyrate (P = 0.005), and total SCFA (P = 0.0008) in faecal samples for the HRM+HAMSB diet compared
with the entry diet, and a significant increase in propionate (P = 0.02) and butyrate (P = 0.04) for the HRM+HAMSB diet compared with the HRM diet (Figure 2). There was no significant difference between the entry and HRM diets for any of the SCFAs measured (P > 0.05), and between the entry and washout diets for any of the SCFAs measured (P > 0.05).

**miRNA expression changes**

miR-17-92 cluster miRNA levels were examined in the rectal biopsy specimens, as these were altered with butyrate treatment in previous studies *in vitro* (18). Two miRNAs that were not altered by butyrate *in vitro* were examined for comparison (18); miR-21, an oncogenic miRNA, and miR-16, a miRNA that is generally abundantly and ubiquitously expressed in normal tissue (26). Levels of miR-17-92 cluster miRNAs increased with the HRM diet compared with the entry diet, but not with the HRM+HAMSB diet. The increased expression of miRNAs within the miR-17-92 cluster with the HRM diet versus the entry diet was significant for miR-19a (P = 0.04) and miR-19b (P = 0.007), and approaching significance for miR-20a (P = 0.08) (Figure 3A). This rise in miR-17-92 miRNA levels with the HRM diet alone was approximately 30% (Figure 3B). Conversely, in the HRM+HAMSB diet, the miR-17-92 cluster miRNA levels were lower than with the HRM diet alone (approximately 20%), which was significant for miR-17 (P = 0.005), miR-19a (P = 0.04), miR-20a (P = 0.003), miR-19b (P = 0.02), and miR-92a (P = 0.02). There was no significant difference between the entry and HRM+HAMSB diet for any of the miR-17-92 cluster miRNAs (P > 0.05), and no significant difference between the entry and washout diets for any of the miR-17-92 miRNAs (P > 0.05). miR-16 appeared stably expressed regardless of the intervention (P > 0.05 for all comparisons) (Figure 3C). There was a significant increase in miR-21 with the HRM diet compared with the entry diet (P = 0.03), and a trend towards an increase with the HRM+HAMSB compared with the entry diet (P > 0.05) (Figure 3C). There was no
significant difference between the HRM and HRM+HAMSB diets for miR-21 (P > 0.05); thus high red meat appeared to alter miR-21 levels, but resistant starch supplementation had little protective effect.

**miR-17-92 target gene changes**

miR-17-92 cluster miRNAs target genes that are important in cell cycle control, including the cell cycle inhibitor *CDKN1A* (target of miRs 17 and 20a) and the pro-apoptotic genes *PTEN* (target of miRs 17, 19a, 19b, and 20a) and *BCL2L11* (target of miRs 17, 18a, 20a, and 92a) (27-30). The influence of the diet-induced changes in miR-17-92 cluster miRNA levels on these target genes was investigated in the rectal biopsy samples. There was a trend towards decreased *CDKN1A*, *PTEN* and *BCL2L11* mRNA levels with the HRM diet compared with the entry diet, which was statistically significant for *CDKN1A* (P = 0.04) (Figure 4A). For *PTEN* and *BCL2L11*, the HRM+HAMSB diet was not significantly different from the entry diet (P > 0.05) or HRM diet (P > 0.05); for *CDKN1A* there appeared to be decreased mRNA levels with the HRM+HAMSB diet compared with the entry diet (P = 0.02). *CDKN1A* and *BCL2L11* mRNA levels appeared lower with the washout diet than with the entry diet; however, this was not statistically significant (P > 0.05)

**Cell Proliferation**

A PCNA assay was used as a proliferation marker in the fixed rectal biopsies. The HRM diet increased proliferation compared with the entry diet (P = 0.02) (Figure 4B). Proliferation with the HRM+HAMSB diet appeared intermediate between the HRM diet and the entry diet, and not significantly different from either the entry diet (P > 0.05) or HRM diet (P > 0.05). Proliferation with the washout diet was significantly higher than with the entry diet (P = 0.02). There was a significant effect of treatment order; consuming the HRM diet first
produced significantly higher proliferation compared with consumption of the HRM+HAMSB diet first (P = 0.04), and compared with consumption of the HRM diet as the second treatment (P < 0.01) (Figure 4C).
DISCUSSION

This randomised cross-over trial is the first to examine the effects of an HRM diet and butyrylated resistant starch supplementation on miRNA expression in rectal mucosa of healthy volunteers. In a novel finding, high red meat intake was shown to alter miRNA levels in rectal mucosa tissue, while HAMSB could mitigate some of these changes. High red meat intake significantly increased rectal mucosa levels of miR-17-92 cluster miRNAs and miR-21, which are both elevated in CRC (16, 17). The miR-17-92 cluster has been designated oncomir-1 (31), and can promote proliferation and angiogenesis, inhibit differentiation, and sustain cell survival (29, 30). Elevated miR-17-92 levels have been associated with invasion and metastasis of CRC cells (32), and poorer survival (33). miR-19a and b in particular are key oncogenic determinants (29, 30), and both were significantly elevated with the HRM diet compared with the entry diet. miR-21 has similarly been classed as oncogenic, and can also induce tumourigenesis, invasion and metastasis (34-36). Elevated miR-21 in CRC has been linked to poorer survival and therapeutic outcomes (37).

Although HRM intake increases CRC risk, butyrylated resistant starch can potentially ameliorate some of these effects. Rodent studies have shown that resistant starch can raise colonic butyrate levels, alter gut microbiota abundance, reduce adenocarcinoma formation in response to a carcinogen, and attenuate red meat-induced DNA damage (5, 11, 22, 38). In this human study, supplementation with butyrylated resistant starch significantly raised faecal butyrate levels. The study identified a novel mechanism by which resistant starch can be beneficial for bowel health, with some of the miRNAs that were elevated in rectal tissue with the HRM diet alone reduced and restored to baseline levels with resistant starch supplementation. In particular, miR-17-92 miRNA levels were significantly lower when the
red meat diet was supplemented with resistant starch. miR-21 and miR-16 levels remained similar in the red meat diets irrespective of resistant starch supplementation. The persistent elevation of miR-21 with red meat intake warrants further investigation to determine any impact on CRC risk.

As miRNAs can simultaneously target hundreds of mRNAs, even small expression changes can have important cellular effects (39). miR-17-92 and miR-21 promote proliferation (18, 29, 30, 34, 35), and examination of target gene expression and cell proliferation provided preliminary evidence regarding the possible influence of detected miRNA changes on cellular function. The increased miR-17-92 miRNA levels with the HRM diet were associated with a decrease in mRNA levels of target genes, particularly the cell cycle inhibitor $CDKN1A$. Through target gene regulation, the increase in miR-17-92 miRNAs and miR-21 with the HRM diet may contribute to the corresponding increase in cell proliferation. Resistant starch supplementation appeared unable to completely restore proliferation to baseline, which could be associated with the miR-21 levels that remained elevated compared with the entry diet. Other regulatory factors, including other miRNAs, may also be involved. It should be noted that the length of the washout period may have been insufficient for these outcomes; proliferation after the washout was significantly higher than after the entry diet, for example, indicating a potential carry-over effect. High variability in mRNA levels also limits the ability to draw firm conclusions from these data, and a larger sample size may have been needed for this outcome.

Another study limitation is the identification of correlations that do not necessarily have cause-and-effect relationships; for instance, it is unclear what component of red meat is increasing miR-17-92 and miR-21 levels. Haem iron, for example, has been associated with
altered gene expression and hyperproliferation of colonic epithelium (40), and haem can also play a role in miRNA processing (41); however, any dietary haem uptake is likely limited to surface epithelial cells (40). High fat or high cholesterol diets can also alter miRNA expression in liver cells (42). It is also not conclusively determined what aspect of the resistant starch is protective. As butyrylated resistant starch was used, this is likely to have directly administered further butyrate to the colon; butyrylated resistant starch can also be more effective in reducing carcinogen damage than standard resistant starch (23, 43).

Offering support for the hypothesis that the miRNA changes with resistant starch supplementation may be due to increased butyrate production, is the \textit{in vivo} replication of \textit{in vitro} findings from previous work where the miR-17-92 cluster but not miR-21 or miR-16 responded to butyrate treatment (18).

An important difference between this study and previous \textit{in vitro} work was that it was performed in volunteers with normal rectal mucosa, rather than in CRC cells. Butyrate is a preferred energy source for normal colonic epithelium and assists in normal proliferation; while alternative fuel sources are preferred in CRC cells, and butyrate instead can inhibit proliferation and induce differentiation or apoptosis (9, 44, 45). Observations in carcinogen-treated rats showed that the colon cells responded to high butyrate levels in a manner more similar to cancer cells, with decreased proliferation and enhanced apoptosis (11, 38). In this study, there was a similar regulation of the miR-17-92 cluster by butyrate in healthy rectal cells \textit{in vivo}, as previously shown in CRC cells \textit{in vitro}. This was observed in the context of high red meat, with resistant starch restoring miRNA levels to those of the entry diet. While there was no significant carry-over effect at the end of the washout period for any miRNA measured, for participants who had the HRM diet first, it is possible that residual red meat effects at the start of the washout period may have reduced the extent to which the resistant
starch decreased miR-17-92 miRNA levels, potentially leading to an under-representation of the degree of attenuation.

This study presents the first evidence in humans that high red meat and butyrylated resistant starch have opposing effects on miRNA levels in rectal mucosa. Several studies have examined the effect of dietary components in other in vivo models. Examination of the miRNA response in rats fed diets containing corn or fish oil with pectin or cellulose and injected with a carcinogen or saline control particularly demonstrated a novel role for fish oil in protecting the colon from carcinogen-induced miRNA dysregulation, rather than a role for fibre (46, 47). Shah et al (2011) did however demonstrate that various dietary combinations and carcinogen exposure modulated a number of miRNAs, including miR-17-92 cluster miRNAs and miR-21 (47).

The oncogenic miR-17-92 cluster was shown to be differentially regulated by dietary factors that increase or decrease CRC risk, and this may explain, at least in part, the respective risk profiles of high red meat and resistant starch. Although the HRM diet increased miR-17-92 cluster miRNA levels in rectal mucosa, with downstream consequences, addition of butyrylated resistant starch to the HRM diet restored miR-17-92 levels to baseline. While the red meat intake during the trial may exceed levels consumed by many in the general population, red meat intake in developed countries is substantial. Total meat consumption in the USA, European Union, and the developed world has continued to increase from 1961 to 2003; nearly doubling in the EU and increasing 1.5-fold in the USA (48). In the USA, per capita total loss-adjusted meat consumption in 2004 was 154 g per day (48). The quantity of resistant starch used in the trial could be realistically applied to the general population. Long term resistant starch supplementation in select populations has been shown to be feasible
(49), and there has been a recent expansion in commercially available foods with increased resistant starch content (50). The findings in this study support increased resistant starch consumption as a means of reducing risk associated with an HRM diet.
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Fig 1: CONSORT diagram of participant flow for the high red meat and resistant starch trial

HRM: red meat diet; HRM+HAMS: red meat and resistant starch diet.
Fig 2: Faecal butyrate, acetate, propionate, and total SCFA levels of participants in the high red meat and resistant starch trial

Faecal samples collected at the end of each four-week diet (* P < 0.05, ** P < 0.01, *** P < 0.001). The mean ± SEM of the 23 participants is shown for each diet. Entry: entry diet; HRM: red meat diet; HRM+HAMSB: red meat and resistant starch diet.
Fig 3: miR-17-92, miR-16 and miR-21 levels in rectal biopsies from participants in the high red meat and resistant starch trial
Rectal biopsies collected at the end of each four-week diet (\* P < 0.05, \** P < 0.01).

Expression measured by real-time RT-PCR and normalised to RNU6B levels. (A) miR-17-92 miRNA levels shown for each diet. (B) Summary of miR-17-92 levels for the intervention diets, presented as percent change from entry diet. (C) miR-16 and miR-21 levels shown for each diet. The mean ± SEM of the 23 participants is shown for each diet. Entry: entry diet; HRM: red meat diet; HRM+HAMSB: red meat and resistant starch diet.
Fig 4: Gene expression and cell proliferation in rectal biopsies from participants in the high red meat and resistant starch trial

Rectal biopsies collected at the end of each four-week diet (* P < 0.05). (A) CDKN1A, PTEN, and BCL2L11 mRNA levels shown for each diet. mRNA levels measured by real-time RT-PCR and normalised to ACTB and B2M levels. (B) Cell proliferation shown for each diet,
measured by proliferating cell nuclear antigen (pCNA) assay. (C) Cell proliferation by
treatment group (Group 1 had HRM diet first, Group 2 had HRM+HAMSB diet first). The
mean ± SEM of the 23 participants is shown for each diet. Entry: entry diet; HRM: red meat
diet; HRM+HAMSB: red meat and resistant starch diet.