Original Paper

Role of Red Meat and Resistant Starch in Promutagenic Adduct Formation, MGMT Repair, Thymic Lymphoma and Intestinal Tumourigenesis in *Msh2*-Deficient Mice

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Key Words

Red meat · Resistant starch · O⁶-methyl-2-deoxyguanosine · Mismatch repair · Methylguanine methyltransferase

Abstract

Red meat may increase promutagenic lesions in the colon. Resistant starch (RS) can reduce these lesions and chemically induced colon tumours in rodents. *Msh2* is a mismatch repair (MMR) protein, recognising unrepaired promutagenic adducts for removal. We determined if red meat and/or RS modulated DNA adducts or oncogenesis in *Msh2*-deficient mice. A total of 100 *Msh2*^{-/-} and 60 wild-type mice consumed 1 of 4 diets for 6 months: control, RS, red meat and red meat + RS. Survival time, aberrant crypt foci (ACF), colon and small intestinal tumours, lymphoma, colonic O⁶-methyl-2-deoxyguanosine (O⁶MeG) adducts, methylguanine methyltransferase (MGMT) and cell proliferation were examined. In *Msh2*^{-/-} mice, red meat enhanced survival compared to control ($p < 0.01$) and lowered total tumour burden compared to RS ($p < 0.167$). *Msh2*^{-/-} mice had more ACF than wild-type mice ($p < 0.014$), but no colon tumours developed. *Msh2*^{-/-} increased cell proliferation ($p < 0.001$), lowered DNA O⁶MeG adducts ($p < 0.143$) and enhanced MGMT protein levels ($p < 0.001$) compared to wild-type mice, with RS supplementation also protecting against DNA adducts ($p < 0.01$). No link between red meat-induced promutagenic adducts and risk for colorectal cancer was observed after 6 months' feeding. Colonic epithelial changes after red meat and RS consumption with MMR deficiency will differ from normal epithelial cells.

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Introduction

Diet has long been known to play a pivotal role in determining the risk for colorectal cancer (CRC). Recent global statistics published by the WHO International Agency for Research on Cancer identifies CRC as the third most diagnosed cancer worldwide, with over 1.36 million cases in 2012 [1]. The report also identifies developed countries such as Australia and Europe with the highest incidence rates, while low-income countries in continents such as Africa having the lowest. Several decades ago, Burkitt [2] recognised that the component of a Western diet containing low dietary fibre and highly refined carbohydrates could be the most important factor attributed to high rates of CRC in developed countries. Not long thereafter, Armstrong and Doll [3] identified an association between red meat consumption and cancer, particularly CRC. Currently, the World Cancer Research Fund identifies red meat and processed red meats as a substantial risk factor for the development of CRC, whereas high dietary fibre, grains and starchy compounds are probably protective [4].

The mechanisms by which red meat might initiate or promote CRC are still being debated. Such processes include: oxidative stress and hyperproliferation by heme [5]; saturated fat promoting tumour formation via secondary bile acid production and lipid peroxidation [6]; protein fermentation by luminal microbiota generating toxic products [7]; carcinogen formation with high-temperature cooking of red meat [8], and lastly, endogenous and exogenous production of alkylating N-nitroso compounds (NOC) that induce alkyl adducts in the DNA [9]. Rodent models incorporating chemically derived alkylating agents can generate large amounts of DNA O⁶-methyl-2-deoxyguanosine (O⁶MeG) adducts in colonic epithelial cells and are directly linked with an increase in aberrant crypt foci (ACF) and colorectal tumours [10, 11]. The first line of defence against these lesions involves the O⁶-methylguanine DNA-methyltransferase (MGMT) repair protein sequestering the O⁶MeG adduct in a suicide reaction. Without MGMT repair, the DNA polymerase complex recognises unrepaired O⁶MeG adducts incorrectly, resulting in GC→AT transition mutations, after which other repair complexes including mismatch repair (MMR) are recruited [12]. A diet with high amounts of red meat in humans is demonstrated to generate significant NOC in faecal content, and this correlates with alkyl adduct formation within exfoliated rectal epithelial cells [13]. In rodents, short-term consumption of dietary red meat can increase colonic O⁶MeG adducts compared to a non-meat protein source [9]. The production of known promutagenic alkyl adducts in the colon by red meat consumption may play a defining role in the oncogenesis of the colon and might act as a biomarker for red meat intake and the associated cancer risk.

Resistant starch (RS) is the component of starch delivered to the colon and fermented by the resident bacteria. It is thought that RS may protect against cancer through mechanisms associated with metabolic products of anaerobic bacterial fermentation in the colon [14]. Studies in rodents have shown associations between faecal short chain fatty acid (SCFA) production by RS and protection against hyperproliferation, preneoplastic markers and CRC [15–17]. In addition, rodents fed a high-protein diet display increased colonic DNA strand breaks, particularly with red meat consumption, but RS could protect against their formation [18–20]. More recently, RS has been identified as an inhibitor of red meat-induced promutagenic O⁶MeG adducts in the mouse, an effect that correlated with faecal butyrate levels [9]. In human trials, RS has shown to reduce colonic proliferation in the colon of CRC patients [21] and healthy individuals consuming a diet with high amounts of red meat [22]. However, due to inadequate RS doses, the effects on epithelial kinetics and adenoma prevention in human trials are somewhat conflicting [23, 24]. RS demonstrates potential for reducing the risk for CRC associated with a Western lifestyle, particularly when consuming a diet with high amounts of red meat. To date, the combination of red meat and RS has not been studied regarding CRC risk at later time points of the oncogenesis pathway, such as precancerous lesions and tumours.

A majority of studies to date use the carcinogen azoxymethane (AOM), an alkylating agent that generates high levels of promutagenic adducts in the distal colon. Therefore, chemical carcinogenic exposure in rodent models of CRC is not an ideal representative of natural dietary carcinogenic exposures. An alternative model for studying dietary effects on CRC are genetically modified rodents that already exhibit an increased susceptibility to CRC. Knockout mice deficient in the *Msh2* protein lack MMR capability and, as a consequence, generate more colonic tumours when exposed to AOM [25]. Although several genetic mouse models of cancer prevention with nutritional compounds have been used, the *Msh2* knockout mouse has not been studied extensively [26]. In the human setting, loss of MMR underlies hereditary non-polyposis CRC (HNPCC), or Lynch syndrome, a common familial inherited form of CRC. HNPCC patients demonstrate increased rates of CRC compared to the normal population as well as earlier onset of disease [27]. In particular, HNPCC individuals with an *Msh2* mutation have a higher age-specific cumulative risk for CRC of 48% by the age of 70 compared to the other common mutations *MLH1* (41%) and *Msh6* (12%) [28]. The *Msh2* knockout mouse represents a relevant and potentially valuable model of study, particularly in relation to red meat-induced adducts and tumour outcome.

It is known that red meat is a risk factor for colorectal oncogenesis, and that RS has the capacity to reduce that risk. Consequently, we determined if feeding red meat to mice deficient in MMR capacity would increase O⁶MeG DNA adducts, proliferation, preneoplastic lesions (ACF) and the risk for colonic neoplasia. We also determined if feeding RS could regulate these effects.

Materials and Methods

Animals and Study Design

Male and female *Msh2*^{-/-} mice initially bred on a 129/OLA background [29] but then backcrossed with C57Bl6J for multiple generations, and their wild-type litter mates, were imported from Australian BioResources (Moss Vale, N.S.W., Australia). A total of 100 *Msh2*^{-/-} and 60 wild-type mice were used in the dietary study. All mice were placed into cages according to gender, with a maximum of 5 per cage. They were randomly divided into dietary groups and the feeding study was strictly under controlled conditions of 22 ± 2°C (SD), 80 ± 10% humidity and 12-hour light/dark cycle. Mice were fed ad libitum and weighed once weekly throughout the entire study. The duration of dietary treatment was designed for 6 months, or until euthanasia attributed to illness. The original strain of *Msh2* knockout mice demonstrate 1/3 survival at 19 weeks [29]. *Msh2*-deficient mice backcrossed with C57Bl6J for multiple generations demonstrate a better survival rate of 2/3 at 19 weeks [30], i.e. double that of the original mouse strain. Therefore, due to the unexpected high rate of early lymphoma and small intestinal tumour development in several mice observed in the current study, an additional 36 *Msh2*^{-/-} mice were included for analysis of cancer and precancerous end points. Research with animals was conducted according to the Australian code for the care and use of animals for scientific purposes. The Flinders University of South Australia Animal Welfare Committee approved all experimental procedures (ethics approval number 809/12).

Diets were based on the American Institute of Nutrition (AIN) diet AIN-76 with some modifications (table 1). A total of 4 dietary interventions were investigated: control, red meat, RS and red meat + RS. Red meat was cooked on a gas BBQ plate at the lowest temperature setting, dried overnight and ground to a powder before mixing in with the dry ingredients. Red meat was used at 25% dry weight, which is within the range of a typical Western diet [9]. Saturated fat content of the red meat final preparation was 10% and analysed by standard lipid extraction with a mixture of chloroform:methanol (1:1, v/v) according to the method of Daugherty and Lento [31]. Lard was used in the non-red meat groups to control for saturated fat content of the red meat diet. Casein (Inpak Foods) was added at 20% of the diet and used as a non-meat protein source in the control and RS diets. High-amylose maize starch (HAMS; Hi-maize 260) was used as the RS source (type RS2) at 10% of the diet and was supplied by Ingredion™. The content of RS in HAMS is approximately 50% [17]; therefore, a total of 5% RS was in the final diet preparations. All

Table 1. Composition of experimental diets (g/100 g of diet)

	Control	RS	Red meat	Red meat + RS
Casein	20	20	–	–
Meat	–	–	25	25
Corn starch	41.72	31.64	39.25	29.17
Hi-Maize™	–	10	–	10
Sucrose	10.95	10.95	10.95	10.95
Sunflower seed oil	17.66	17.74	17.68	17.76
Lard ^a	2.55	2.55	–	–
α-Cellulose	2	2	2	2
L-cysteine	0.3	0.3	0.3	0.3
Choline	0.17	0.17	0.17	0.17
Minerals ^b	3.5	3.5	3.5	3.5
Vitamins ^b	1	1	1	1
DL-methionine	0.15	0.15	0.15	0.15
Total	100	100	100	100

^a Lard was added to the control diets to balance for saturated fat content of the red meat.

^b AIN-76 standard vitamin and mineral mixtures.

dry ingredients were mixed thoroughly before the wet ingredients were combined. Approximately 500–800 ml of distilled water was blended into the diet mixture before being compressed into pellets. Final pelleted food was stored in air-tight containers at -20°C and replaced in the cage feeders every 1–2 days.

Specimen Collection, Storage and Tissue Preparation

At the end of the 6-month feeding experiment, or when signs of deteriorating illness were evident, the mice were humanely euthanized by CO₂ asphyxiation and cervical dislocation. The entire length of the colon was resected, faecal contents removed, and cut open longitudinally before being affixed to Hi-bond™ membrane paper. Colons were submerged in a 10% buffered formalin solution containing 3.6% formaldehyde for 24 h and transferred to 70% ethanol for storage. The small intestine was examined and any suspected tumours were removed, placed into 10% buffered formalin solution and transferred to 70% ethanol at 24 h. The diagnosis of lymphoma included but was not limited to: significant weight loss (10–15%), deterioration of overall mouse health before death/euthanasia, rapid shallow breathing, enlarged thymus, spleen or liver, and/or lung tumour.

Small Intestinal Tumour Histology

Suspected small intestinal tumours were processed through gradient alcohols and xylene before being embedded in paraffin wax. A 5-µm section was taken and rehydrated in gradient alcohols before staining with Harris haematoxylin and eosin for visualisation under light microscope. Tumours were classified as adenomas or adenocarcinomas. Adenomas were characterised by expansion of the mucosal layer, reduction in goblet cell number, moderate loss of mucosal architecture by glandular growth and dilated crypts. Adenocarcinomas were identified when there was severe distortion of cytological and glandular architecture, loss of cell polarity, prominent cellular atypia and invasion through the muscularis mucosae.

Analysis of Colonic ACF

Excess mucus and debris were washed from the colon with 0.9% solution of saline and gently wiped clean with a cotton tip before incubation in 0.4% solution of methylene blue diluted with 0.9% saline solution for approximately 1–2 min. Visualisation of ACF was performed under a dissecting microscope and identified as having crypts with large openings, unusual shape and stained darker than the normal surrounding crypts. ACF were classified as small (≤3 aberrant crypts), large (≥4 aberrant crypts) and total ACF.

Immunohistochemical Quantification of Proliferation, O⁶MeG Adducts and MGMT Repair

After ACF analysis, 0.5 cm of colorectal tissue was cut from the distal colon and from the proximal portion where the 'herringbone' pattern of the proximal colon meets the flatter, middle colon. Both small intestinal tumours and the colonic tissue segments were dehydrated through gradient alcohols and xylene before being embedded in paraffin wax. Distal and proximal sections of 4 µm were used to quantify cell proliferation with an antibody against the nuclear proliferating antigen Ki-67 (AbCam SP6; cat. No. ab16667) and O⁶MeG DNA adducts with the primary monoclonal antibody (clone EM 2–3; Squarix Biotechnology). Methods for immunohistochemical (IHC) protocols are described as per Winter et al. [9], with the exception of proximal colon tissue which used a primary O⁶MeG antibody incubation temperature of 4°C instead of room temperature. The sum of intensity of O⁶MeG adduct staining for red meat and RS treatment was normalised relative to control diet levels. Slides were coded and a total of 20 intact colonic crypt columns were counted by a single observer who was blinded to dietary groups.

Quantification of MGMT protein was performed with a mouse monoclonal antibody (SPM287; cat. No. ab54306) diluted 4-fold with phosphate-buffered saline (PBS; pH 7.4) in combination with a mouse-on-mouse polymer linking kit (Covance). Briefly, distal and proximal 4-µm sections were rehydrated with gradient alcohols, endogenous enzymes blocked with 3% H₂O₂ in 50% ethanol for 15 min before antigen retrieval with 0.01 mol/l citrate buffer (pH 6.5) for 1 h in a 2100 antigen retriever (PickCell Laboratories). Preblocking with normal serum block (Covance) for 15 min was performed before overnight incubation with the diluted MGMT antibody at 4°C. Slides were washed in PBS (3 × 2 min), followed by 30 min incubation with boost solution (Covance), washed again in PBS (3 × 2 min) before 20 min incubation with mouse-on-mouse polymer-HRP solution (Covance). Positive cells were visualised with DAB chromogen and substrate (Covance), and tissue sections were counterstained with Harris haematoxylin for observation under a light microscope. Proliferation was measured as positive Ki-67 cells per crypt column. O⁶MeG and MGMT were calculated as the sum of pixel intensity (red to blue ratio) measured by computer image analysis (detailed in Winter et al. [9]). Slides were coded, and a total of 20 intact colonic crypt columns were counted by a single observer who was blinded to dietary groups.

Statistical Analysis

Cumulative survival of *Msh2*^{-/-} mice is presented as Kaplan-Meier curves and generated using the original 100 *Msh2*^{-/-} mice. Overall survival between all groups and the control versus red meat groups was analysed with the log-rank (Mantel-Cox) test. To avoid variation of adduct staining attributed to aging [32], IHC measurements of the colonic epithelia and final mouse bodyweights were analysed in 12 mice from each dietary group (both *Msh2*^{-/-} and wild type) that survived beyond 5 months. Data for O⁶MeG and MGMT were standardised to control the diet for each wild type and *Msh2*^{-/-} mouse before comparison tests were carried out. Univariate analysis (three-way ANOVA) was used to identify effects and interactions of RS, red meat or genotype on final bodyweight, proliferation, O⁶MeG and MGMT. Due to the very small number of *Msh2*^{-/-} mice that developed ACF, an effect of RS or red meat was analysed using a generalised linear model (Wald χ^2 test). Diet effects on small intestinal tumour incidence were analysed using a generalized linear model: ordinal logistic (χ^2 test) and, for lymphoma incidence, crosstabs (Pearson's χ^2 test). Overall tumour burden identified as the mean number of tumours per mouse was compared initially using one-way ANOVA with Bonferroni correction to adjust for multiple comparisons, followed by two-way ANOVA to identify the effects and interaction of red meat and RS on total tumour burden. Observed power for total tumour burden as the major endpoint was 0.245, and for ACF the observed power was 0.111. All other observed power values for IHC variables measured were 0.816 for proliferation, 0.725 for O⁶MeG adducts and 1.0 for MGMT. Data are represented as the mean ± standard error (SE). Statistical significance was accepted at the $p < 0.05$ level.

Results

*Red Meat Improves Overall Survival and Demonstrates a Trend for Protection against Tumour Burden in *Msh2*^{-/-} Mice*

Overall food consumption was the same for each wild-type and *Msh2*-deficient mice (data not shown). Final bodyweights of both *Msh2*^{-/-} and wild-type mice were not significantly affected by dietary treatment; however, *Msh2*^{-/-} mice had significantly lower bodyweights

Fig. 1. *Msh2* knockout survival. Cumulative survival of *Msh2*^{-/-} mice over a 6-month dietary treatment (26 weeks) presented as Kaplan-Meier curves showing comparisons of all dietary treatments (**a**) and control versus red meat diet (**b**).

Table 2. Incidence (%) of colonic ACF, small intestinal (SI) tumours, lymphoma and mean total tumour burden in *Msh2*^{-/-} mice

	Experimental diets				Dietary effects, p value ²	
	control (n = 35)	RS (n = 31)	red meat (n = 28)	red meat + RS (n = 31)	RS effect	red meat effect
SI tumours	20.6	30	10.7	22.6	0.194	0.412
Lymphoma	38.2	33.3	28.6	35.5	0.625	0.520
Small ACF	11.42	12.90	14.28	16.12	0.754	0.620
Large ACF	0	3.225	7.142	3.225	0.987	0.286
Total ACF	11.42	19.35	21.42	19.35	0.486	0.592
Total tumour burden ¹	0.69±0.13	0.84±0.13	0.43±0.11	0.81±0.16	0.249	0.343

¹ Data represented as mean ± SD.

² Tested using two-way ANOVA.

(35.51 ± 1.15) compared to wild-type mice (40.61 ± 1.12; $p < 0.002$). Comparison of overall survival for *Msh2*^{-/-} mice between all 4 diet groups did not reach statistical significance ($p = 0.057$; fig. 1a). However, when comparing mice consuming control diet versus red meat diet, there was a statistically significant improvement in survival for those mice consuming the red meat diet ($p < 0.01$; fig. 1b). Loss of *Msh2* was associated with increased mean ACF per mouse from no ACF at all in the wild-type mice to 0.18 ± 0.05 ($p < 0.05$). There was no significant effect of any diet on small, large or total ACF. Overall, very few ACF were observed in any of the dietary groups (table 2). *Msh2*^{-/-} mice consuming a red meat diet had lower rates of both small intestinal tumours and lymphoma, although the difference was not significant (table 2). Analysis of the mean tumour burden per mouse (using one-way ANOVA) by combining small

Fig. 2. **a** Total tumour burden of *Msh2*^{-/-} mice combining small intestinal tumours and lymphoma. Analyses were carried out using one-way ANOVA with the Bonferroni correction to adjust for multiple comparisons. No significant differences were observed. Data are represented as mean $\pm$ SE. Representative images of H&E staining of normal-appearing epithelium and small intestinal adenocarcinoma (**b**) and a thymic lymphoma (**c**).

intestinal tumours and lymphoma (fig. 2) showed a trend for reduction in tumours with red meat compared to RS ($p < 0.167$). Analysis by two-way ANOVA showed there was no significant effect of red meat ($p < 0.343$) or RS ($p < 0.249$) on total tumour burden. No colorectal tumours (adenomas or invasive cancers) were observed in any mice.

*RS Protects against *Msh2*^{-/-} Hyperproliferation in the Distal Colon*

Proliferation rates in the distal colon for wild-type and *Msh2*^{-/-} mice are shown in figure 3a. The level of positive Ki-67 cells per crypt was significantly higher in *Msh2*^{-/-} mice compared to their wild-type litter mates ($p < 0.001$). Hyperproliferation in *Msh2*^{-/-} mice was suppressed by addition of RS to the diet, although this did not reach significance ($p < 0.202$; fig. 3b). However, this effect of RS was not seen in the proximal colon (data not shown). In contrast, RS significantly increased proliferation in the proximal colon compared to diets without RS for wild-type mice ($p < 0.05$), although this did not reach significance in *Msh2*^{-/-} mice ($p = 0.163$; data not shown).

Fig. 3. a Effect of genotype, red meat and RS consumption on proliferation rates in the distal colon by three-way ANOVA. **b** Interaction of RS consumption and genotype by two-way ANOVA on distal colon proliferation. **c** Representative images showing the hyperproliferative state of distal colon in *Msh2*^{-/-} mice and its reduction with RS supplementation. Data are represented as means $\pm$ SE.

RS and Msh2 Deficiency Protects against O⁶MeG DNA Adducts

Results of O⁶MeG DNA adducts normalised to control are presented in figure 4a. Red meat did not significantly change O⁶MeG adducts in the distal colon compared to mice consuming non-red meat diets ($p < 0.533$). RS diets protected against O⁶MeG adducts compared to non-RS diets ($p < 0.01$). Loss of *Msh2* did not enhance red meat-induced O⁶MeG

Fig. 4. **a** Main effects of red meat, RS and genotype on the sum of O⁶MeG DNA adduct staining intensity normalised to control in distal colon cells. Representative images of O⁶MeG DNA adduct IHC staining of the distal colon for RS versus non-RS diets (**b**) and wild-type versus *Msh2*^{-/-} mice (**c**). Data are represented as mean ± SE.

adducts (fig. 4a). In fact, O⁶MeG adducts demonstrated a trend of reduction in *Msh2*^{-/-} mice compared to wild-type mice ($p < 0.143$). No significant changes in O⁶MeG adduct accumulation were observed in the proximal colon (data not shown).

MGMT Protein Expression Is Enhanced on an MMR-Deficient Background but Decreases with RS Supplementation

To determine if changes in MGMT repair might be the mechanism by which *Msh2*^{-/-} and RS supplementation display altered DNA adducts in mice, MGMT protein expression was measured in the distal colon. *Msh2*^{-/-} mice displayed significantly higher MGMT protein expression in the distal colon compared to wild-type mice ($p < 0.001$). MGMT protein expression was significantly decreased in mice consuming RS diets compared to mice consuming diets without RS ($p < 0.00001$). Interaction tests between RS and genotype (fig. 5b) showed that MGMT activity was enhanced in *Msh2*^{-/-} mice only consuming an RS diet, although this did not reach significance ($p < 0.118$).

Fig. 5. Main effects of red meat, RS and genotype on the sum of MGMT DNA adduct staining intensity normalised to control in distal colon cells (**a**) and interaction of RS consumption and genotype by three-way ANOVA (**b**). Data are displayed as mean $\pm$ SE. RM = Red meat; WT = wild type; KO = knockout.

Discussion

We have shown for the first time that the consumption of red meat demonstrates a trend for protection against lymphoma and small intestinal tumours in MMR-deficient mice, and that MMR deficiency has a protective effect on the formation of DNA promutagenic alkyl adducts in the distal colon. We have also shown for the first time that *Msh2*-deficient mice generate an adaptive response of MGMT protein expression. Moreover, we have confirmed previous data showing that RS consumption in normal mice can protect against the formation of these lesions in the distal colon. Loss of *Msh2* capacity increased cell proliferation and spon-

taneous ACF formation in the colon. Our results also show that a diet high in RS can impede *Msh2*^{-/-}-associated hyperproliferation of the distal colon and return the epithelium to a more normal proliferative state. Yet, none of the observed changes with the dietary interventions had a significant effect on ACF or colon tumours within the 6-month duration of this experiment.

Contrary to our original hypothesis, red meat consumption in an *Msh2*-deficient mouse did not enhance unrepaired O⁶MeG DNA adducts and was not a significant risk factor for ACF formation. However, DNA O⁶MeG adduct accumulation was lower in *Msh2*-deficient mice compared to wild-type mice. What this means for CRC risk cannot be assessed since no animals developed CRC, certainly not within the 6-month duration of the experiment. Since MMR capacity is deficient in these mice, it seemed possible that *Msh2*-deficient mice might produce an adaptive response of MGMT repair to account for the loss of *Msh2*, thereby reducing unrepaired O⁶MeG adduct load. Indeed, when we measured MGMT protein expression, we found a significant increase in MGMT expression in *Msh2*-deficient mice, indicating that an adaptive MGMT response is present in these mice when MMR is inefficient. Furthermore, there was a strong significant reduction in MGMT protein expression and reduction in O⁶MeG adducts with RS supplementation, suggesting that RS primarily prevents these adducts from forming, thus negating the need for MGMT repair. This could be due to the bulking qualities of RS in the lumen reducing the contact time of potential carcinogens and mutagens with the epithelial cell layer [14]. Furthermore, RS consumption when on a high-protein diet switches fermentation from protein to carbohydrate, changing the metabolic profile [9, 32], and, therefore, could be reducing the alkylating capacity of the luminal contents by changing the microbiota diversity. Further studies measuring faecal metabolites such as NOC and analysing the microbiota profile might explain these findings. It is also important to note that the MGMT antibody used in the IHC analysis in the current study measures both active and inactive forms of MGMT. Therefore, MGMT activity levels may be different, even though changes in protein expression were identified. Due to inadequate tissue sampling in the mice, we were unable to measure MGMT activity, but future analysis of MGMT activity in *Msh2*-deficient mice is justified.

Although we detected changes in DNA adduct accumulation and enhanced MGMT repair with *Msh2* deficiency, no effect on colonic proliferation or precancerous lesions (ACF) were identified, although a trend toward increasing large ACF was observed with red meat consumption ($p < 0.286$). In humans, carriers of the MGMT Ile143Val polymorphism (which dampens MGMT activity) show a significant interaction of red meat and processed red meat intake (>56 g/day) and increased risk for CRC [33]. This suggests that accumulation of red meat-induced alkyl adducts due to ineffective MGMT activity might be important for the development of CRC in MMR-proficient individuals. However, carriers of the MMR gene defect do not display the same risk for CRC after the consumption of a diet with high amounts of red meat [34, 35]. Voskuil et al. [34] found an increased risk for CRC within a sporadic CRC population consuming red meat ranging from <4 times a week to >7 times a week ($p = 0.08$), but no such risk amongst the HNPCC population ($p = 0.26$). Meat consumption >7 times a week in this study demonstrated an OR of 4.1 (95% CI 0.7–23) for sporadic CRC cases and only 0.4 (95% CI 0.1–2.2) for HNPCC cases. These data, together with our MGMT and ACF results in *Msh2*^{-/-} mice, suggest that the effect of red meat and MGMT repair in colonic epithelial cells with an MMR-deficient background might not be the same as in the general population. There appears to be an undetermined component of red meat, or apparent change in MGMT repair mechanism in MMR-deficient mice, that might protect against alkyl adduct formation as a result of MMR loss. Transition mutations in the *K-ras* oncogene are known to be induced by alkylating agents [36] and could be explored in future studies to determine if these observed changes in adducts and MGMT effect *K-ras* mutation frequency in MMR-deficient mice

consuming red meat and/or RS diets. The significance of the DNA O⁶MeG adduct acting as a marker for exposure of red meat on a normal genetic background, and not necessarily as a biomarker of CRC risk, seems to be likely. However, the model used here may not have been entirely adequate to confirm this theory.

Unexpectedly, we observed a trend toward reduction in small intestinal tumours and lymphoma in MMR-deficient mice consuming a red meat diet, and a significantly enhanced survival rate in these mice. *Msh2*^{-/-} mice develop intestinal tumours similar to HNPCC in older mice but spontaneous lymphomas occur at an earlier age (1/3 survival at 19.5 weeks) [29]. We hypothesised that red meat might act as an initiating ‘carcinogen’ (since we have shown that it increases adduct formation [9]), and that colonic neoplasms would develop (similar to chemical alkylation administration with AOM) as the MMR defect would make them more susceptible to the effects of red meat. Our results show that the *Msh2*^{-/-} mouse is not a good model in determining CRC risk as a result of red meat consumption. It is possible that heterozygous *Msh2* mice may be more informative. In fact, HNPCC patients are heterozygous carriers of an MMR gene defect and do not normally develop cancers in childhood. In contrast, children inheriting a homozygous MMR gene defect from families of affected HNPCC patients (MMR deficiency syndrome) have a distinct pathology of gastrointestinal cancers, hematologic malignancies and neurological tumours all occurring in early childhood [37]. In addition to MMR, *Msh2* is also important for somatic intrachromosomal recombination [38], an important process in the protection against tumours via a properly functioning immune system. The role of red meat consumption and its apparent protective effect on malignancies associated with homozygous MMR deficiency is noteworthy. Whether red meat may be enhancing the immune system via other pathways within *Msh2*^{-/-} mice, thereby reducing the risk for malignancies as a result of defective immune responses is unknown. More studies elucidating the effects of red meat on tumourigenesis and the immune system in an MMR-deficient background is needed to validate these findings.

Our findings support previous data [9, 39, 40] emphasising how RS can reduce the proliferation and DNA alkyl adducts in the distal colon of wild-type rodents. We have also shown that functioning *Msh2* protects against hyperproliferation and ACF formation in the colon, and that RS consumption protects against colonic hyperproliferation caused by the genetic defect of MMR. Previous research has shown that fermentation of RS in the rodent colon, both with and without red meat protein, generates significantly high levels of SCFAs in the colonic lumen, including butyrate [9, 19, 20]. Antineoplastic properties identified in vitro and in vivo highlight the butyrate’s ability to increase the removal of highly damaged colon cells via apoptosis, as well as reducing cellular proliferation of the distal colon to allow for repair processes [41–43]. These effects of butyrate imply that it is responsible for reducing ACF and colon tumours in genetically normal rodents [39, 44] although no such reduction was evident in non-carcinogenic models of spontaneous CRC [32]. Reduction of hyperproliferation by RS was not seen in the proximal colon, and this could be due to the rapid formation of SCFAs once RS reaches the proximal colon from the caecum, with a steady decline in SCFAs further along the colon [45]. Butyrate is the primary energy source for colorectal cells and such a rise in SCFAs in this region of the colon might explain the increase in proliferation, and not necessarily a link to increased risk for cancer. Our ACF findings in *Msh2*^{-/-} mice support data from the CAPP2 trial of HNPCC patients where RS consumption did not reduce CRC risk, although doses of RS in that study were arguably low at approximately 15 g of RS (in the 30 g/day of HAMS administered) [24, 46]. Maintaining colonic proliferation via RS fermentation may not reduce ACF formation induced by loss of MMR. In the current study, *Msh2*^{-/-} mice did not live long enough for a substantial amount of ACF to form and longer intervention times may be required. RS may protect against CRC in chemical carcinogenic rodent models, but its chemoprotective capacity against cancer generated by a Western diet remains questionable.

There were a few limitations in this study that affected the potential to produce definitive results. Firstly, the power of the study was not adequate to identify changes in tumour burden and ACF formation. This was likely due to very small numbers of ACF forming in the colon of *Msh2*-deficient mice, and a low number of remaining mice at the conclusion of the study due to extracolonic tumours forming. Secondly, the length of the study was inadequate to allow sufficient ACF and colon tumours to form; therefore, longer dietary intervention times would be needed in the future using a larger sample of *Msh2*^{-/-} mice. Lastly, using mice was an essential component of this study (due to the nature of the genetic knockout utilised); however, as a consequence tissue sampling was extremely limited. Therefore, important analyses were not feasible but may have proved more informative and supported our current findings.

Contrary to our hypotheses, this study has revealed an unexpected protective effect of MMR deficiency on colon O⁶MeG adduct formation, likely as a consequence of an enhanced MGMT response in those mice. This study also reveals a trend of reduction in lymphoma and small intestinal cancer and an enhanced survival rate in *Msh2*^{-/-} mice consuming red meat. However, we did not show a link between colonic promutagenic adducts and red meat consumption, or any associated risk for CRC. Although RS suppressed hyperproliferation of the distal colon as a result of MMR deficiency, we did not identify any link between lowered proliferation rates and ACF formation. Consequently, the CRC risk associated with changes in colonic proliferation by RS fermentation in the context of MMR remains questionable. Variations in epithelial markers after red meat and RS consumption on a normal genetic background do not always behave in a similar fashion with MMR-deficient capacity. These findings highlight the potential implications for dietary guidelines given to individuals at high risk of developing CRC, particularly those with an inherited genetic defect in MMR.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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