Dietary Manipulation of Oncogenic MicroRNA Expression in Human Rectal Mucosa: A Randomized Trial


Abstract
High red meat (HRM) intake is associated with increased colorectal cancer risk, while resistant starch is probably protective. Resistant starch fermentation produces butyrate, which can alter microRNA (miRNA) levels in colorectal cancer cells in vitro; effects of red meat and resistant starch on miRNA expression in vivo were unknown. This study examined whether a 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 randomized cross-over design, 23 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. Fecal butyrate increased with the HRM+HAMSB diet. Levels of oncogenic mature miRNAs, including miR17–92 cluster miRNAs and miR21, increased in the rectal mucosa with the HRM diet, whereas the HRM+HAMSB diet restored miR17–92 miRNAs, but not miR21, to baseline levels. Elevated miR17–92 and miR21 in the HRM diet corresponded with increased cell proliferation, and a decrease in miR17–92 target gene transcript levels, including CDKN1A. The oncogenic miR17–92 cluster is differentially regulated by dietary factors that increase or decrease risk for colorectal cancer, and this may explain, at least in part, the respective risk profiles of HRM 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 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 (HRM) 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 colorectal cancer risk (1). Plausible mechanisms include inducing DNA strand breaks and enhancing promutagenic DNA adduct formation (3, 4). HRM consumption has also been linked to gut microbiome changes and inflammation (5, 6). In contrast, dietary fiber probably protects against colorectal cancer, with systematic review evidence identifying a dose–response relationship, and 10% decreased risk per 10 g fiber intake per day (1). Interventional studies provide less conclusive evidence, and longer-term trials and higher fiber levels may be needed to reproduce effects from observational studies (7).

One protective mechanism for fiber is the production of fermentation products, particularly the short-chain fatty acid (SCFA) butyrate (1). Butyrate is a histone deacetylase inhibitor, with antitumorigenic effects (8–12). Aberrant microRNA (miRNA) expression contributes to colorectal cancer development (13–15), with miRNAs such as miR21 and the miR17–92 cluster of miRNAs often increased in colorectal cancers and possessing oncogenic properties (16, 17). We have shown that butyrate can modulate miRNA expression in colorectal cancer cells in vitro (18). The miR17–92 cluster, comprising miR17, miR18a, miR19a, miR20a, miR19b, and miR92a, was significantly decreased with butyrate. This decrease may be partially responsible for the antiproliferative effects of butyrate, with addition of miR17–92 mimics reversing this and increasing proliferation; miR19a and miR19b in particular were key promoters of proliferation (18). Through epigenetic mechanisms, butyrate may be able to reverse the miRNA dysregulation observed in colorectal cancer (18).

Higher colonic butyrate levels can be achieved with resistant starch supplementation compared with other fiber 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 fecal butyrate compared with standard high amylose maize starch (P < 0.0001; refs. 20 and 21). In rodents, resistant starch supplementation to an HRM 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 fiber may modify DNA adduct formation in the context of HRM 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 randomized cross-over design, markers of colorectal cancer risk were measured in healthy human volunteers who undertook 2 4-week intervention diets, an HRM diet, and an HRM diet supplemented with butyrylated resistant starch (HRM+HAMSB; StarPlus, National Starch and Food Innovation, Bridgewater, NJ). It was hypothesized that regulation of miRNA expression may partially explain some of the chemoprotective effects of resistant starch and the increased colorectal cancer risk associated with HRM intake.

Materials and Methods

Study design

Dietary interventions were explained during clinic visits. The study consisted of 2 intervention periods of 4 weeks each, preceded by a 4-week run-in (entry) period and separated by a 4-week washout period. Volunteers were randomized 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; ref. 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 prepacked 20 g sachets, with 2 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 2 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, fiber, 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 fecal samples, and miRNA expression changes, target gene levels, and cell proliferation were measured from rectal mucosa samples. Fecal and rectal pinch biopsy specimens were obtained at the end of each 4-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 3 days of each 4-week dietary period. Food diaries were entered into Foodworks Professional 7 nutritional calculation software (Xyris Software) by a dietitian, to calculate energy and macronutrient intake based on Australian food composition tables and food manufacturers’ data.

Fecal collection was conducted by participants for the last 24 hours of each dietary period. Samples were homogenized in 3 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) within an Agilent 6890N Network GC system (Agilent). 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 4 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). Additional biopsies were formalin fixed. Biopsies stored in RNAlater were used for quantitation of miRNA and target gene mRNA levels. Each biopsy was homogenized in 0.5 mL TRIzol Reagent (Ambion), and total RNA extracted according to the manufacturer’s instructions. RNA was quantified using a Nanodrop-8000 spectrophotometer (Nanodrop Technologies).

miRNA expression analysis was conducted by relative quantitation real-time RT-PCR using TaqMan miRNA assays (Applied Biosystems). 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 x TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems; assay IDs: miR17: 000393, miR18a: 002422, miR19a: 000395, miR20a: 000580, miR19b: 000396, miR92a: 000430, miR16: 000391, miR21: 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), 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 x TaqMan gene expression master mix (Applied Biosystems; assay IDs: CDKN1A: Hs00355782_m1, PTEN: Hs02621230_s1, BCL2L1: 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).

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). Deyparaffinized 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) was placed on slides (1:500 dilution) and incubated overnight, followed by incubations with a Murine Ultra-Streptavidin HRP Detection Kit (Covance Laboratories). Visualization was performed using 3,3'-diaminobenzidine chromagen and substrate buffer (Covance Laboratories), with slides counterstained with hematoxylin. 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.). Statistical analyses were performed in IBM SPSS Statistics 22 (IBM SPSS Inc.) 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 analyzed 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 4-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 (Fig. 1). Two participants withdrew before commencing the interventions; 1 because of unrelated medical problems, and 1 because of intolerance of the first rectal biopsy. At study completion, 23 participants had received both intervention diets (17 males and 6 females, ages 50–75 years), and data from these participants were analyzed. There were no major complications, and all 23 volunteers tolerated sample collection and the interventions, except 1 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). Fiber and starch intake were decreased in the HRM diet compared with the entry diet (P < 0.0001 and P = 0.02, respectively) and HRM+HAMSB diets (P < 0.0001 and P = 0.02, respectively).

**Fecal 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 fecal 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 (Fig. 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

miR17–92 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); miR21, an oncogenic miRNA, and miR16, a miRNA that is generally abundantly and ubiquitously expressed in normal tissue (26). Levels of miR17–92 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 miR17–92 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 miR17–92 cluster with the HRM diet versus the entry diet was significant for miR19a ($P = 0.04$) and miR19b ($P = 0.007$), and approaching significance for miR20a ($P = 0.08$; Fig. 3A). This rise in miR17–92 miRNA levels with the HRM diet alone was approximately 30% (Fig. 3B). Conversely, in the HRM+HAMSB diet, the miR17–92 cluster miRNA levels were lower than with the HRM diet alone (approximately 20%), which was significant for miR17 ($P = 0.005$), miR19a ($P = 0.04$), miR20a ($P = 0.003$), miR19b ($P = 0.02$), and miR92a ($P = 0.02$). There was no significant difference between the entry and HRM+HAMSB diet for any of the miR17–92 cluster miRNAs ($P > 0.05$), and no significant difference between the entry and washout diets for any of the miR17–92 miRNAs ($P > 0.05$). miR16 seemed stably expressed regardless of the intervention ($P > 0.05$ for all comparisons; Fig. 3C). There was a significant increase in miR21 with the HRM diet compared with the entry diet ($P = 0.03$), and a trend toward an increase with the HRM+HAMSB compared with the entry diet ($P > 0.05$; Fig. 3C). There was no significant difference between the HRM and HRM+HAMSB diets for miR21 ($P > 0.05$); thus, HRM seemed to alter miR21 levels, but resistant starch supplementation had little protective effect.

miR17–92 target gene changes

miR17–92 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 proapoptotic genes PTEN (target of miRs 17, 19a, 19b, and 20a) and BCL2L11 (target of miRs 17, 18a, 20a, and 92a; refs. 27–30). The influence of the diet-induced changes in miR17–92 cluster miRNA levels on these target genes was investigated in the rectal biopsy samples. There was a trend toward decreased CDKN1A, PTEN, and BCL2L11 mRNA levels with the HRM diet compared with the entry diet, which was statistically significant for CDKN1A ($P = 0.04$; Fig. 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 seemed to be decreased mRNA levels with the HRM+HAMSB diet compared with the entry diet ($P = 0.02$). CDKN1A and BCL2L11 mRNA

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**Figure 1.** CONSORT diagram of participant flow for the HRM and resistant starch trial. HRM, red meat diet; HRM+HAMSB, red meat and resistant starch diet.
levels seemed 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 ($P = 0.02$; Fig. 4B). Proliferation with the HRM+HAMSB diet seemed 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 ($P < 0.01$; Fig. 4C).

Discussion

This randomized 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, HRM intake was shown to alter miRNA levels in rectal mucosa tissue, whereas HAMSB could mitigate some of these changes. HRM intake significantly increased rectal mucosa levels of miR17–92 cluster miRNAs and miR21, which are both elevated in colorectal cancer (16, 17). The miR17–92 cluster has been designated oncomir-1 (31), and can promote proliferation and angiogenesis, inhibit differentiation, and sustain cell survival (29, 30). Elevated miR17–92 levels have been associated with invasion and metastasis of colorectal cancer cells (32), and poorer survival (33). Elevated miR17–92 levels have been associated with invasion and metastasis of colorectal cancer cells (32), and poorer survival (33). Elevated miR21 in colorectal cancer has been linked to poorer survival and therapeutic outcomes (37).

Although HRM intake increases colorectal cancer 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 fecal 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 restored to baseline levels with resistant starch supplementation. In particular, miR17–92 miRNA levels were significantly lower when the HRM diet was supplemented with resistant starch. miR21 and miR16 levels remained similar in the HRM diets irrespective of resistant starch supplementation. The persistent elevation of miR21 with red meat intake warrants further investigation to determine any impact on colorectal cancer risk.

As miRNAs can simultaneously target hundreds of mRNAs, even small expression changes can have important cellular effects (39). miR17–92 and miR21 promote...
proliferation (18, 29, 30, 34, 35), and examination of target gene expression and cell proliferation provided preliminary evidence about the possible influence of detected miRNA changes on cellular function. The increased miR17–92 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 miR17–92 miRNAs and miR21 with the HRM diet may contribute to the corresponding increase in cell proliferation. Resistant starch supplementation seemed
unable to completely restore proliferation to baseline, which could be associated with the miR21 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 miR17–92 and miR21 levels. Heme iron, for example, has been associated with altered gene expression and hyperproliferation of colonic epithelium (40), and heme can also play a role in miRNA processing (41); however, any dietary heme 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 carcinogenic 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 in vivo replication of in vitro findings from previous work where the miR17–92 cluster but not miR21 or miR16 responded to butyrate treatment (18).

An important difference between this study and previous in vitro work was that it was performed in volunteers with normal rectal mucosa, rather than in colorectal cancer cells. Butyrate is a preferred energy source for normal colonic epithelium and assists in normal proliferation; whereas alternative fuel sources are preferred in colorectal cancer 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 miR17–92 cluster by butyrate in healthy rectal cells in vivo, as previously shown in colorectal cancer cells in vitro. This was observed in the context of HRM, with resistant starch restoring miRNA levels to those of the entry diet. Although 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 miR17–92 miRNA levels, potentially leading to an underrepresentation of the degree of attenuation.

This study presents the first evidence in humans that HRM 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 fiber (46, 47). Shah and colleagues (47) did however demonstrate that various dietary combinations and carcinogen exposure modulated a number of miRNAs, including miR17–92 cluster miRNAs and miR21 (47).

The oncogenic miR17–92 cluster was shown to be differentially regulated by dietary factors that increase or decrease colorectal cancer risk, and this may explain, at least in part, the respective risk profiles of HRM and resistant starch. Although the HRM diet increased miR17–92 cluster miRNA levels in rectal mucosa, with downstream consequences, addition of butyrylated resistant starch to the HRM diet restored miR17–92 levels to baseline. Although 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 United States, European Union (EU), and the developed world has continued to increase from 1961 to 2003; nearly doubling in the EU and increasing 1.5-fold in the United States (48). In the United States, 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.

Disclosure of Potential Conflicts of Interest
N A Kennedy has received speakers’ bureau honoraria from MSD and has provided expert testimony (support to attend meetings) for Ferring, Abbvie, Shire, Warner Chilcott, and Norgine. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: K J Humphreys, M A Conlon, G P Young, D L Topping, A R Bird, L Cobiac, M Z Michael, R K Le Leu
Development of methodology: M Z Michael, R K Le Leu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K J Humphreys, G P Young, N A Kennedy, M Z Michael, R K Le Leu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K J Humphreys, G P Young, R K Le Leu
Writing, review, and/or revision of the manuscript: K J Humphreys, G P Young, D L Topping, Y Hu, J Winter, A R Bird, L Cobiac, N A Kennedy, M Z Michael, R K Le Leu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K J Humphreys, J Winter
Study supervision: G P Young, D L Topping, Y Hu, M Z Michael

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Karen J. Humphreys, Michael A. Conlon, Graeme P. Young, et al.


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