Diet-associated inflammation modulates inflammation and WNT signaling in the rectal mucosa, and the response to supplementation with dietary fibre

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Running title: Dietary inflammatory index and WNT signalling

Keywords: dietary inflammatory index, WNT signalling, dietary fibre, colorectal cancer, inflammation

Financial support: This work was supported by an award from the BBSRC Diet and Health Research Industry Club (DRINC) (grant number BB/H005013/1) to JCM, ITJ, NJB and SK. IM was funded by a fellowship from Northumbria NHS Foundation Trust. The authors acknowledge further support from the Newcastle University Centre for Ageing and Vitality, which is funded by the Medical Research Council and BBSRC as part of the cross-council Lifelong Health and Wellbeing Initiative (grant no. MR/L016354/1), and from the Wellcome Trust. Further funding was awarded by the Wellcome Trust Broadening Our Horizons scheme to support the collaboration between Newcastle University and the University of South Carolina.

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Conflict of interest statement: The authors declare no potential conflicts of interest.

Disclosure: Dr. James R. Hébert owns controlling interest in Connecting Health Innovations LLC (CHI), a company that has licensed the right to his invention of the Dietary Inflammatory Index (DII®) from the University of South Carolina in order to develop computer and smart phone applications for patient counselling and dietary intervention in clinical settings. Dr. Nitin Shivappa and Dr. Michael Wirth are employees of CHI. The subject matter of this paper will not have any direct bearing on that work, nor has that activity exerted any influence on this project.

Clinical Trial Registry: The DISC Study is registered with ClinicalTrials.gov (NCT01214681).

Word Count: 4756

Number of Tables: 4

Number of Figures: 2

Abbreviations:

ANOVA: analysis of variance

β-catenin: beta-catenin

β-coefficient: beta coefficient

BMI: body mass index

CCPS: crypt cell proliferative state

CI: confidence intervals

CRC: colorectal cancer

CRP: C-reactive protein

DII: Dietary inflammatory index

DISC Study: Dietary Intervention, Stem cells and Colorectal Cancer Study

E-DII: energy-adjusted DII

GLM: general linear model

IBD: inflammatory bowel disease

MAPK: Mitogen Activated Protein Kinase

NFκB: Nuclear Factor kappa B

NIH-AARP: National Institutes of Health-American Association of Retired Persons

PD: polydextrose

RCT: randomised controlled trial

RS: resistant starch

ROS: reactive oxygen species

SCFA: short-chain fatty acid

SEM: standard error of the mean

SFRP: secreted frizzled-related protein

STAT3: Signal transducer and activator of transcription 3

UC: ulcerative colitis

ABSTRACT

Inflammation drives colorectal cancer (CRC) development and CRC risk is influenced by dietary factors, including dietary fibre. Hyperactive WNT signalling occurs in CRC and may regulate inflammation. This study investigated i) relationships between the inflammatory potential of diet, assessed using the Energy-adjusted Dietary Inflammatory Index (E-DII™), and markers of WNT signalling, and ii) whether DII status modulated the response to supplementation with two types of dietary fibre.

Seventy-five healthy participants were supplemented with resistant starch (RS) and/or polydextrose (PD) or placebo for 50 days. Rectal biopsies were collected pre and post-intervention and used to assess WNT pathway gene expression and crypt cell proliferation. E-DII scores were calculated from food frequency questionnaire data. High-sensitivity C-reactive protein (hsCRP) and faecal calprotectin concentrations were quantified.

hsCRP concentration was significantly greater in participants with higher E-DII scores (least square means (LSM) 4.7 vs. 2.4mg/L, P=0.03). Baseline E-DII score correlated with *FOSL1* (β= 0.503, P=0.003) and *WNT11* (β=0.472, P=0.006) expression, after adjusting for age, gender, BMI, endoscopy procedure and smoking status. *WNT11* expression was more than two-fold greater in individuals with higher E-DII scores (LSM 0.131 vs. 0.059, P=0.002). Baseline E-DII modulated the effects of PD supplementation on *FOSL1* expression (P=0.04).

More pro-inflammatory diets were associated with altered WNT signalling and appeared to modulate the effects of PD supplementation on expression of *FOSL1*.

This is the first study to investigate relationships between the E-DII and molecular markers of WNT signalling in rectal tissue of healthy individuals.

INTRODUCTION

Approximately half of colorectal cancer (CRC) cases are attributable to 'modifiable' lifestyle factors e.g. obesity and diet(1, 2). For example, there is "probable" evidence higher consumption of foods containing dietary fibre lowers CRC risk(3). However, because foods and nutrients are not consumed in isolation, it is important to assess diet healthfulness holistically when investigating relationships with disease-related outcomes(4).

Inflammation modulates CRC risk(5-8), and individuals with inflammatory bowel disease (IBD) are at increased risk of CRC(9). The Dietary Inflammatory Index (DII®) quantifies the inflammatory potential of the whole diet(10), and comprises 45 food parameters, including 36 anti-inflammatory components e.g. dietary fibre(10). The DII has been validated in various cohorts and shown to correlate with the expression of inflammatory markers e.g. C-reactive protein (CRP), IL-6 and IL-10(11-15). Furthermore, more pro-inflammatory DII scores are associated with greater risk of all-cause mortality(16) and of cancers(17) including CRC(18). A systematic review and meta-analysis of nine studies revealed that individuals in the highest DII category of exposure had 40% increased risk of CRC compared with those in the lowest category, translating to a 7% increase in CRC risk for each one-point increase in DII score(18). The underlying mechanisms linking DII and CRC risk are not fully understood, but are likely to include effects of the inflammation-related components of the diet on insulin sensitivity, the gut microbiome, local inflammation (which promotes cell proliferation and mutagenesis(19)) and on the production of reactive oxygen species (ROS)(7, 18), as well as modulation of molecular pathways e.g. WNT signalling.

The WNT signalling pathway regulates cellular processes such as proliferation that contribute to the maintenance of homeostasis and tissue self-renewal in the large intestine(20). Aberrant WNT signalling in CRC includes abnormal expression of β-catenin and adenomatous polyposis coli (*APC*)(21). Furthermore, WNT genes e.g. *WNT11* are upregulated in colonic tissue from ulcerative colitis (UC) patients (22). Recent evidence suggests that WNT signalling may influence the inflammatory state via cross-talk with pathways including Nuclear Factor kappa B (NFκB) and Mitogen Activated Protein Kinase (MAPK)(23). WNT signalling may also regulate the activity

of inflammatory pathways, e.g. β-catenin inhibits NF-κB signalling(24), and the expression of inflammatory cytokines and chemokines, e.g. *WNT5A* induces IL-1 and IL-6(25-27). In addition, inflammatory cytokines regulate mucosal WNT signalling via Protein Kinase B (AKT) signalling(28).

The WNT pathway plays an important role in the link between diet, adiposity and physical activity, and gastrointestinal cancers including CRC(29, 30) and several dietary factors modulate WNT pathway activity(31, 32). We have shown that higher adherence to the World Cancer Research Fund (WCRF) Cancer Prevention Recommendations, which includes anti-inflammatory components of the DII such as dietary fibre, was associated with altered expression of WNT pathway components(33). Adherence to the sub-recommendation on dietary fibre intake was associated with significantly lower rectal expression of β-catenin and of *WNT11*(33). Higher dietary fibre intake protects against CRC(3), and short-chain fatty acids (SCFA) produced by dietary fibre fermentation, primarily butyrate, are chemoprotective and exert anti-inflammatory effects, some of which may be mediated via modulation of WNT signalling(34, 35). In the Dietary Intervention, Stem cells and Colorectal cancer (DISC) Study, we supplemented healthy individuals with two types of dietary fibre, resistant starch (RS) and polydextrose (PD), and observed downregulation of β-catenin, *c-MYC*, *SFRP1* and *SFRP2* in the rectal mucosa(36).

Taken together, the evidence suggests that the WNT pathway mediates the effects of diet, including perhaps its inflammatory potential, on CRC risk. Therefore, this study had two aims: i) to test the hypothesis that diet-associated inflammation is related to WNT pathway activity by investigating relationships between DII score and expression of WNT pathway components in the rectal mucosa of healthy individuals; and ii) to investigate whether the inflammatory potential of habitual diet modulated the response to supplementation with RS and/or PD in the DISC Study. We also investigated relationships between DII score and crypt cell proliferative state (CCPS) as a functional outcome of WNT signalling, and biomarker of CRC risk(35, 36).

MATERIALS AND METHODS

The DISC Study Participants

This study used data and samples from the DISC Study (ClinicalTrials.gov Identifier: NCT01214681), a randomised, placebo-controlled dietary intervention that investigated the effects of two types of dietary fibre (RS and PD) on markers of CRC risk(36, 37). The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Newcastle and North Tyneside Research Ethics Committee (REC No. 09/H0907/77). Healthy participants were recruited from gastroenterology out-patients departments at North Tyneside General Hospital, North Shields, UK and Wansbeck General Hospital, Ashington, UK between May 2010 and July 2011. Written informed consent was obtained from all participants.

Dietary intervention

Participants were supplemented with RS and/or PD or placebo for 50 days in a 2 x 2 factorial design. At least one week after their first endoscopy appointment, participants were randomised to one of four intervention groups: RS (23 g Hi-maize® 260, Ingredion™, Food Innovation), PD (12 g of Litesse® Ultra™ DuPont™ Danisco®), RS and PD or double placebo (12 g of Maltodextrin (RS placebo) and 23 g of Amioca starch (PD placebo)). Randomisation was stratified by endoscopy procedure (flexible sigmoidoscopy or colonoscopy).

Sample collection

Phenotypic data (e.g. height and body weight) and biological samples were collected pre- and post-intervention. Rectal mucosal biopsies were collected at endoscopy (colonoscopy or flexible sigmoidoscopy for baseline samples and rigid sigmoidoscopy for post-intervention samples) using Biobite Biopsy forceps (Medical Innovations) from the mid-rectum (10cm from the ano-rectal verge). For the collection of stool samples, participants were given a sealable bucket pot, a disposable bedpan, two ice packs (to be frozen prior to sample collection) and a cool bag. Participants stored samples in cool bags containing the frozen ice packs. Pre-intervention stool samples were collected at least seven days after the endoscopy appointment and picked up by the research team from the participants' homes, and post-intervention samples were brought by the participant to the second endoscopy appointment. Samples were divided into aliquots and stored at -80°C until analysis.

Measurement of inflammatory markers

High-sensitivity C-reactive protein (hsCRP) in serum was quantified at Newcastle Laboratories, Freeman Hospital (Newcastle upon Tyne, UK) from blood samples collected in one 5ml BD Vacutainer® SST™ II Advance tube with gold hemogard closure (Becton Dickinson, UK). Faecal calprotectin was quantified in extracts from 100mg of stool using the Faecal Sample Preparation Kit (Calpro AS, Lysaker, Norway). Prior to preparation of faecal extracts, samples were defrosted overnight and mixed using Stomacher®80 Biomaster (Seward Ltd, Worthing, UK). Extracts were diluted 1:20 in sample dilution buffer and used to quantify faecal calprotectin using the Calprolab™ Calprotectin ELISA (ALP) kit (Calpro AS). Optical density was read after 40 minutes incubation with enzyme substrate solution on a FLUOstar® Omega microplate reader (BMG Labtech Ltd, Aylesbury, UK) operated by BMG Omega software version 1.20.

Expression of WNT pathway components

RNA was extracted from rectal mucosal biopsies using the RNeasy Mini Kit (Qiagen) using five 3mm glass beads (VWR) and QiaShredders (Qiagen) for tissue disruption and homogenisation, respectively. cDNA was synthesised from 1µg RNA using the QuantiTect Reverse Transcription Kit (Qiagen). The expression of 12 WNT pathway genes and two reference genes (*18S* and *β2M*) was quantified by quantitative PCR (qPCR) using the StepOnePlus™ Real Time PCR system (Applied Biosystems). These target genes were selected by reviewing the literature to identify WNT genes that were a) implicated in colorectal carcinogenesis (selection criterion 1) and b) whose expression is modified by butyrate (a product of dietary fibre fermentation; selection criterion 2) (Supplementary Table 1). In addition, *APC* was chosen due to its key role in the WNT pathway and in CRC. We have found that the expression *18S* and *β2M* reference genes is stable in rectal mucosal samples(36).

Quantification of *CCND1, c-MYC* and *SFRP1* was performed using primers designed and optimised by Dr. Nigel Belshaw and Dr. Wing Leung (Quadram Institute, Norwich, UK) (Supplementary Table 2). For these three genes, together with two reference genes (*18S* and *β2M*), qPCR reactions contained 5µl ImmoMix™ (2x) (Bioline, UK), 0.1 µl MgCl2 (50mM) (Bioline, UK), 1µl BSA (10mg/ml) (Ambion, UK),

0.2µl ROX Reference Dye (50x) (Invitrogen, UK), 0.06µl SYBR Green (100x) (Invitrogen, UK), 0.6µl RNase-free water, 0.02µl each of forward and reverse primers (100µM) and 3µl of cDNA. The programme was run for a 10 minute activation step at 95°C followed by 40 cycles of 30 seconds each, denaturation at 95°C, annealing at 60°C and extension at 72°C. For the remaining nine genes, qPCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) and QuantiTect primer assays (Qiagen, Supplementary Table 3), with reactions containing 15µl of master mix and 5µl of the sample cDNA. The programme was run for a 15 minute activation step at 95°C followed by 40 cycles of 15 seconds denaturation at 94°C, 30 seconds annealing at 55°C and 30 seconds extension at 72°C. All samples were run in duplicate. Each plate contained pre- and post-intervention samples for each participant and representatives from each intervention group. Data collection was during the extension stage and melting curve analysis was performed. Gene expression data are expressed as adjusted values ($2^{-\Delta Ct}$ x 10,000) relative to the geometric mean of *18S* and *β2M* reference genes(38).

Assessment of rectal crypt cell proliferative state (CCPS)

Rectal CCPS was assessed in whole, microdissected, Schiff reagent-stained crypts(37). Briefly, Carnoy's-fixed rectal mucosal biopsies were hydrated in 50% ethanol, followed by 25% ethanol, for 10 minutes each at room temperature. Biopsies were then hydrolysed in 1M HCl for 10 minutes at 60°C and stained with Schiff reagent (Surgipath™) for one hour at room temperature. The Schiff reagent was replaced with 1ml of 45% acetic acid and whole crypts were microdissected using an Olympus SZ40 dissecting microscope and Leica CLS 150X light source. On a microscope slide with a drop of 45% acetic acid, rows of individual crypts (bases of the crypts facing upwards) were teased apart using fine gauge hypodermic needles (25G x 5/8" Terumo®, Belgium) and covered and sealed with a cover slip (Surgipath®, Leica, UK). Ten intact crypts were selected at random and each divided into ten equal compartments longitudinally, starting from the base of the crypt. The number of mitotic cells in each compartment was counted, and from this the proportion of mitotic cells in the upper half of the crypt was calculated, as well as crypt width and length measurements, from which crypt volumes were calculated.

Quantification of faecal SCFA concentrations

SCFA concentrations were quantified by gas chromatography using pivalic acid as an internal standard as described previously(39). Briefly, 1ml 20mM pivalic acid and 5ml water were added to 1g of faecal sample, mixed thoroughly and centrifuged at 5000xg for 5min. 0.250ml saturated oxalic acid solution was added to 0.5ml of the supernatant and incubated at 48°C for 1 hour. This was centrifuged at 16 000xg for 5min and the supernatant fraction was used for analysis as described previously(40).

Calculation of energy-adjusted DII (E-DII)

Habitual diet was assessed at baseline using a food frequency questionnaire (FFQ) adapted from that used in the EPIC – Norfolk Study (version 6, CAMB/PQ/6/1205)(41), asking participants for their average consumption of foods over the last year. The inflammatory potential of diet was assessed by calculating the DII scores and energy-adjusted DII (E-DII™) scores(10). Dietary intakes of 29 food components (alcohol, beta-carotene, carbohydrates, cholesterol, fibre, total fat, iron, trans fatty acids, folate, energy, magnesium, monosaturated fatty acids, niacin, polyunsaturated fatty acids, protein, retinol, riboflavin, saturated fatty acids, selenium, thiamine, vitamin B6, vitamin B12, vitamin C, vitamin D, vitamin E, zinc, onions, garlic, tea) were included in the calculation(10). Intake from foods only, not supplements, was included in DII calculations. A total E-DII score was calculated by adding the scores for each of the 29 food parameters, and expressed per 1000 kilocalories (4.187 MJ) consumed. Higher E-DII scores indicate more pro-inflammatory diets, whereas lower E-DII scores represent less inflammatory, or more anti-inflammatory, diets.

Statistical analyses

For descriptive statistical analyses, independent sample t-tests and Fisher's exact tests were used for comparisons between the lower and higher E-DII groups. In cross-sectional analyses, multivariable regression models were used to investigate relationships between E-DII and the measured outcomes, with model 2 adjusting for age, gender, endoscopy procedure, BMI and smoking status as covariates. For categorical analyses, participants were divided into a low E-DII (more anti-inflammatory) and a high E-DII (more pro-inflammatory) group by dichotomising at the median E-DII (0.700). Differences in the measured outcomes between the low

and high E-DII groups at baseline were investigated using the ANOVA General Linear Model (GLM) and adjusting for age, gender, endoscopy procedure, BMI and smoking status as covariates. Models were not adjusted for total energy intake because it is one of the components of the DII and is explicitly accounted for in the calculation of E-DII scores.

For the RCT, interactions between E-DII status at baseline and the effects of the dietary intervention (RS and/or PD) on the measured outcomes post-intervention were investigated using the ANOVA GLM, adjusting for pre-intervention measurement, age, gender, endoscopy procedure, BMI and smoking status as covariates. All statistical analyses were performed using IBM® SPSS® Statistics version 25. $P<0.05$ was considered statistically significant.

RESULTS

Participant demographics

Seventy-five healthy participants were recruited to the DISC Study (Table 1). The mean age of participants was 52 years (range 30-80 years) and 53% were female. Most of the participants (97%) were White. For more details, see Malcomson *et al.*(36).

Inflammatory potential of diets of DISC Study participants

The mean E-DII score was slightly pro-inflammatory (0.736 ± 0.253) and E-DII scores ranged from -4.480 to 5.030. Table 1 shows the participants' characteristics according to E-DII group. Participants with more pro-inflammatory diets, i.e. those in the higher-E-DII group, were more likely to be former or current smokers (P= 0.03).

Relationships between E-DII and inflammatory markers

hsCRP concentrations in the higher, more pro-inflammatory, E-DII group were approximately two-fold greater compared with the lower E-DII group (P=0.03) (Table 2). Although faecal calprotectin concentrations were, on average, 32% higher in individuals in the higher E-DII group, the considerable inter-individual variation within groups meant that this difference was not statistically significant (P=0.46). There were no significant relationships between E-DII and faecal calprotectin or hsCRP

concentrations when investigated using the regression models (Supplementary Table 4).

Relationships between E-DII and WNT pathway markers

In the unadjusted multilevel linear regression model, E-DII score was significantly associated with baseline (pre-intervention) rectal expression of *FOSL1* (β=0.414, P=0.01) and *WNT11* (β=0.365, P=0.009) (Table 3). These findings were strengthened in the fully adjusted model (*FOSL1* (β=0.503, P=0.003) and *WNT11* (β=0.472, P=0.006)). Furthermore, participants in the higher E-DII group had more than two-fold higher expression of *WNT11* compared with those in the lower E-DII group (least squares means 0.131 vs. 0.059, P=0.002, Figure 1).

There were no significant associations observed between E-DII and the remaining 10 WNT pathway components (Table 3), nor differences in their expression between the lower and higher E-DII groups (Supplementary Table 5).

Interestingly, there was a weak but significant correlation between rectal mucosal *WNT11* expression and faecal calprotectin concentrations (Spearman's correlation coefficient= 0.362, P=0.01). No such relationship was observed, however, for hsCRP (Spearman's correlation coefficient= 0.142, P=0.33) and there were no significant correlations between rectal *FOSL1* expression and the inflammatory markers measured in this study (hsCRP (Spearman's correlation coefficient= 0.234, P=0.16) and faecal calprotectin (Spearman's correlation coefficient= -0.248, P=0.15)).

Relationships between E-DII and rectal crypt cell proliferation state (CCPS) at baseline

There were no significant associations between E-DII score and total mitoses in the rectal epithelium, proportion of mitoses in the top half of the crypts (CCPS outcomes measured in this study) or crypt dimensions (length, width and volume) (Table 4). In addition, crypt dimensions and rectal CCPS outcomes did not differ between participants with lower and higher E-DII scores (Supplementary Table 6). Furthermore, there were no significant correlations between expression of *FOSL1*

and *WNT11* (that were associated with E-DII (Table 3)), and CCPS outcomes or crypt dimensions (Supplementary Table 7).

Interaction between baseline E-DII and the effects of supplementation with RS and PD on the measured outcomes

The effects of RS and PD on WNT pathway-related outcomes have been published previously(36, 37). In the present study, we investigated whether E-DII scores, derived from habitual diet data assessed at baseline, modulated the response to RS and PD. There were no significant differences in the inflammatory potential of habitual diet (i.e. E-DII score) according to dietary intervention group at baseline (P=0.64) (Supplementary Table 8). We observed a significant interaction effect of E-DII and PD supplementation on post-intervention rectal *FOSL1* expression (P=0.04, Figure 2). Individuals in the higher E-DII group at baseline, with a more pro-inflammatory diet, had a lower post-intervention *FOSL1* expression when given PD compared with those with less inflammatory E-DII scores. In individuals given the placebo, individuals with higher E-DII scores had higher post-intervention *FOSL1* expression compared with those with less inflammatory diets in the lower E-DII group. There were no interaction effects between EDII and RS and/or PD on the other quantified genes or inflammatory and CCPS markers measured (Supplementary Table 9).

DISCUSSION

Chronic inflammation is a key risk factor for CRC by causing mutations, chromosomal alterations and aberrant patterns of DNA methylation which lead to oncogene activation, tumour suppressor inactivation, dysregulated DNA repair and chromosomal instability(42). In addition, both inflammatory state and CRC risk are influenced by environmental and lifestyle factors, especially diet(3, 43). Aberrant WNT signalling occurs early in the tumorigenic process(44) and provides both a selective advantage for the initial clonal expansion, and genetic instability for subsequent tumour progression and malignant transformation(45). WNT signalling is modulated by dietary factors including dietary fibre(34) and there may be cross-talk between WNT signalling and inflammatory pathways(24). The inflammatory potential of individual diets can be assessed using the DII(10); higher DII values indicate a more pro-inflammatory diet and have been associated with increased expression of inflammatory markers(11, 12, 14, 15) as well as greater CRC risk (18). However, little is known about the relationships between DII scores and molecular markers of CRC risk. This study is the first to report associations between DII and WNT pathway activity, CCPS and crypt dimensions in the healthy rectal mucosa, and to explore the potential modulation by habitual DII of the response to supplementation with dietary fibre.

E-DII is associated with expression of *WNT11* and *FOSL1* in the rectal mucosa of healthy adults

We observed significant positive correlations between the E-DII scores and expression of *WNT11* and *FOSL1* in the rectal mucosa of DISC Study participants. WNT11 is a ligand that regulates the activation of both canonical and non-canonical WNT signalling pathways(46) and its expression is induced by WNT pathway activation and by factors such as TGF-β(47). In the intestinal epithelium, WNT11 regulates cell proliferation, intercellular adhesion and migration and, consequently, is implicated in tumorigenesis(48). *WNT11* is upregulated in CRC(49) and is involved in cancer progression(50). Upregulation of *WNT11* in colorectal adenocarcinomas may play a role in colorectal tumourigenesis through stimulation of WNT signalling(49) and greater expression of *WNT11* has been reported in patients with UC(22). In the

present study, a 'less inflammatory diet', as assessed by a lower E-DII score, was associated with reduced *WNT11* expression. When stratifed by age, the difference between E-DII groups remains statistically significant for the younger (<50 years) group only (p=0.03) (Supplementary Table 10). It is probable that the reduction in group sizes coupled with the greater inter-individual variability in participants aged ≥50 years limited our ability to detect the between E-DII groups among older participants. In addition, rectal *WNT11* expression correlated positively with faecal calprotectin, a marker of gastrointestinal inflammation. Interestingly, we have previously reported lower rectal mucosal expression of *WNT11* in participants with greater adherence to the WCRF Cancer Prevention Recommendations(33). Furthermore, adherence to the recommendation to consume ≥25g dietary fibre/ day, an anti-inflammatory component of the DII(10), and to the recommendation to be physically active, were associated with lower *WNT11* expression(33). These findings suggest that *WNT11* may be particularly sensitive to modulation by environmental and lifestyle factors, including diet. Although they will require confirmation in independent studies, our findings suggest that such relationships between lifestyle factors and rectal mucosal markers may be affected by age, which is particularly important as this is the strongest risk factor for CRC. Furthermore, because the molecular characteristics of sporadic CRC cases in early-onset (age <50 years) differ from those developing CRC at an older age(51), and age-dependent effects on other markers of CRC risk have been reported(32, 37, 52), further studies investigating these age-dependent processes are warranted.

Greater expression of *FOSL1* (Fos-related antigen 1 (also known as *FRA-1*)) was also associated with higher E-DII scores, i.e. more inflammatory diets, in the rectal mucosa of healthy individuals. *FOSL1* is a member of the *FOS* oncogene family and a target gene of the WNT pathway. Increased FOSL1 protein and greater β-catenin accumulation occurs in human colorectal adenocarcinomas(53). Interestingly, increased IL-6 secretion as a consequence of activation of signal transducer and activator of transcription 3 (STAT3) signalling promotes *FOSL1* deacetylation in CRC cell lines, resulting in increased *FOSL1* expression. Furthermore, increased FOSL1 protein was observed in cancer tissue from CRC patients, and this correlated with abundance of the pro-inflammatory cytokine IL-6(54). Aberrant *FOSL1* expression has also been reported in patients with mild UC, and expression levels were

positively correlated with concentrations of IL-11 in biopsies from UC patients (55). To our knowledge, this is the first study to report relationships between diet quality and *FOSL1* expression in the rectal mucosa.

E-DII and expression of other WNT signalling genes in the rectal mucosa

In the present study, we did not detect relationships between E-DII and the expression of the other 10 quantified WNT pathway-related genes. As this is the first study to explore such relationships, we could not be specific about which WNT genes would be modulated by E-DII. Since these target genes were selected because of their potential modulation by dietary fibre(36), it is possible that not all are responsive to differences in the inflammatory potential of diet. However, a previous mouse study suggested that high fat diet-induced inflammation was associated with downregulation of *Apc* and increased expression of *Ctnnb1* and target genes e.g. *c-Jun* and *Ccnd1* in the colon(56). In the present study performed in healthy human adults, we observed no relationships between the inflammatory potential of habitual diet and expression of these four genes in the rectal mucosa.

Dietary fibre supplementation may modulate the relationships between E-DII and *FOSL1* in the rectal mucosa

We investigated whether baseline E-DII modulated the effects of supplementing healthy individuals with dietary fibre (provided as RS and/or PD) on WNT pathway-related markers of CRC risk. We observed a significant interaction between E-DII and supplementation with PD on rectal expression of *FOSL1,* in which those with poorer, more inflammatory diets (i.e. higher E-DII scores) had lower post-intervention *FOSL1* expression. Because lower *FOSL1* expression may be associated with lower CRC risk, this finding suggests that those with poorer diets may benefit more from supplementation with PD. The opposite was observed for those given placebo; those with higher E-DII scores had higher post-intervention *FOSL1* expression. To our knowledge, this is the first study to explore whether baseline E-DII modulates the response to a dietary intervention. However, there is evidence of a poorer response to bariatric surgery (smaller weight and fat mass changes) in individuals with more inflammatory baseline DII scores(57). We explored whether the observed differences

in *FOSL1* expression in response to PD supplementation between lower and higher E-DII groups could have resulted from differences in faecal SCFA concentrations. We hypothesised that individuals with poorer diets, indicated by higher E-DII scores, may have lower SCFA concentrations at baseline, which may lead to greater relative change in SCFA with PD supplementation, and therefore respond better to the dietary intervention. However, there were no significant differences between individuals with lower and higher E-DII scores in the faecal concentrations or proportions of SCFAs at baseline (Supplementary Table 11) nor in the change in SCFAs post-intervention. The potential mechanisms underpinning the observed effects, and why these were observed for PD supplementation but not for RS, are unclear. Therefore, further research is warranted to substantiate this novel finding.

Greater CCPS and, especially, a higher proportion of mitotic cells in the top half of the crypt, is a biomarker of CRC risk(58, 59). In the present study, for the first time, we investigated relationships between E-DII score and rectal CCPS in healthy participants but we did not observe any significant relationship. Previous studies suggest that dietary components, such as dietary fibre, that modulate inflammation may mediate CRC risk via effects on cell proliferation(37, 52, 60, 61). Butyrate, produced from bacterial fermentation of dietary fibre, activates T-regulatory cells that block pro-inflammatory T-cells, leading to reduced production of cytokines associated with the stimulation of cell proliferation(62). Chronic inflammation is associated with activation of WNT signalling, induced by the STAT3 pathway, which stimulates cell proliferation in the colorectal epithelium(63). In a mouse model of chronic colitis, supplementation with red raspberries, which contain anti-inflammatory compounds, was associated with reduced expression of WNT pathway components that regulate the cell cycle (*CCND1, c-MYC)* as well as cell proliferation in colonic tissue(64). Furthermore, WNT pathway activity, assessed by the quantification of β-catenin expression, and STAT3 signalling were also reduced by red raspberry supplementation(64).

Strengths and limitations of study

This was a tightly controlled study with careful measurement of exposures, covariates and outcomes. The DISC Study is one of the largest studies assessing a

variety of molecular and functional markers of large bowel health and of CRC risk in the macroscopically-normal rectal mucosa, and the largest RCT investigating these effects of dietary fibre in healthy people. All participants were recruited from the same region (two hospitals in the North East of England) using stringent inclusion and exclusion criteria, such as excluding any participants on anti-inflammatory medication, thus minimising the effects of potential confounders. However, this study is limited by its relatively small sample size and lack of ethnic diversity. Whilst the relatively homogenous population within this study reduces the effects of potential confounders, this may limit the generalisability of findings to other populations, with different dietary patterns, socioeconomic status, education, ethnicity and geographical location.

Estimation of habitual dietary intake using self-reported data from FFQs, which are prone to recall bias and misreporting, was used to calculate E-DII scores. At the individual level, BMI has well-recognised limitations as an index of adiposity. Future studies should investigate potential modifying effects of adiposity on E-DII links with CRC risk. Further, baseline biopsies were collected by two different endoscopy procedures, with different bowel preparation requirements. However, for the RCT, randomisation was stratified according to baseline endoscopy procedure, and this was included as a covariate during statistical analyses. In addition, all of the biopsies were collected from the same anatomical site, so reducing potential confounding. Our use of data from a cross-sectional study means that we cannot attribute causality to the observed relationships between E-DII and expression of WNT pathways genes in the rectal mucosa. Such relationships will need to be confirmed in future intervention studies.

Conclusions

Our findings suggest that the WNT signalling pathway may mediate some effects of inflammatory dietary components on markers of large bowel health in the healthy rectal mucosa. More specifically, more pro-inflammatory diets are associated with greater expression of *FOSL1* and *WNT11,* both of which are more highly expressed in CRC tissue and in tissue from IBD patients. Furthermore, individuals with greater E-DII scores had reduced rectal *FOSL1* expression after PD supplementation.

Expression of both *FOSL1* and *WNT11* has been associated with levels of inflammatory cytokines such as IL-6(47, 54). Interestingly, we observed a weak but significant correlation between rectal *WNT11* expression and the concentration of faecal calprotectin, a local marker of intestinal inflammation. Therefore, *FOSL1* and *WNT11*, putative markers of CRC risk, may be responsive to dietary factors, and may have potential as surrogate endpoints in dietary intervention studies. Since WNT signalling is also modulated by adipose tissue, and obesity-induced inflammation is a risk factor for CRC, further investigations exploring molecular changes in adipose tissue may be of interest(65). Furthermore, investigations into the potential modulation of diet-related inflammation and WNT signalling by obesity and/or body mass change are warranted(29).

To our knowledge, this is the first study to investigate relationships between the inflammatory potential of diet, assessed using the E-DII, and molecular markers in the target tissue (i.e. rectal tissue) of healthy individuals and the first to explore whether E-DII modulates the response to supplementation with dietary fibre. Further investigations, using transcriptome-wide and multi-omic approaches studies, of how the inflammatory potential of habitual diet, assessed using the DII, modulates the response to dietary and other lifestyle interventions are warranted.

ACKNOWLEDGEMENTS

We are very grateful to DISC Study participants without whom this study would have been impossible. We acknowledge the staff at the gastroenterology units at Wansbeck and North Tyneside General Hospitals for their help and support with participant recruitment and study visits. We thank Ingredion™, formerly National Starch, Food Innovation, USA and DuPont™ Danisco®, Finland for supplying the RS and PD, respectively. We are very grateful to Julie Coaker for her very kind assistance with training in the assessment of CCPS.

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TABLES

Table 1 DISC Study participant characteristics at baseline according to E-DII group

Demographics	**Lower E-DII group (≤0.700)**	**Higher E-DII group (>0.700)**	**P-value**
Total *n*	37	38	
Female *n (%)*	24 (65)	16 (42)	0.07
Age (years)	53.0 (1.9)	51.9 (2.1)	0.43
Race/Ethnicity			1.00
White *n (%)*	36 (97)	37 (97)	
Black *n (%)*	1 (3)	0 (0)	
Mixed race *n (%)*	0 (0)	1 (3)	
Endoscopy procedure			0.15
Flexible sigmoidoscopy *n (%)*	22	30	
Colonoscopy *n (%)*	15	8	
Anthropometrics			
Height (m)	1.65 (0.01)	1.68 (0.02)	0.20
Weight (kg)	78.9 (2.5)	87.0 (2.6)	0.59
BMI (kg/m^2)	28.9 (0.8)	31.1 (0.9)	0.33
Waist circumference (cm)	95.7 (2.0)	103.3 (2.1)	0.48
Hip circumference (cm)	106.3 (2.0)	107.8 (1.8)	0.63
Smoking status			**0.03***
Never *n (%)*	24 (65)	14 (37)	
Former[1] *n (%)*	9 (24)	12 (32)	
Current *n (%)*	4 (11)	12 (32)	
E-DII	-0.999 (0.229)	2.425 (0.217)	0.92

Data are presented as means with standard error of the mean in parentheses unless otherwise stated. Independent sample t-tests and Fisher's exact tests were used for comparisons between the lower and higher E-DII groups, *p<0.05. [1]Former smokers include participants who had stopped smoking prior to the start of the DISC Study.

795 *Table 2 Inflammatory markers at baseline according to E-DII group*

Inflammatory marker	**All participants**	**Lower E-DII group (≤0.700)**	**Higher E-DII group (>0.700)**	**P-value**
Faecal calprotectin (mg/kg)	15.5 (54.0)	13.2 (35.7)	17.4 (63.9)	0.46
hsCRP (mg/L)	3.6 (0.5)	2.4 (0.4)	4.7 (0.9)	**0.03***

796 Data for hsCRP are presented as means and standard error of the mean (SEM) in
797 parentheses, independent sample t-test. Data for faecal calprotectin are presented as
798 medians and interquartile ranges in parentheses, Mann-Whitney. *P<0.05.
799

800 *Table 3 Associations between the Dietary Inflammatory Index (E-DII) and expression of*
801 *WNT pathway genes in the rectal mucosa at baseline*

	Model 1			Model 2		
WNT gene	**β coefficient**	**95% CI**	**P value**	**β coefficient**	**95% CI**	**P value**
APC	0.147	-0.066, 0.194	0.33	0.152	-0.086, 0.217	0.39
AXIN2	0.112	-0.112, 0.295	0.37	0.016	-0.207, 0.233	0.91
CCND1	0.074	-16.3, 25.8	0.65	-0.034	-26.6, 22.2	0.86
CTNNB1	0.090	-0.638, 1.357	0.47	-0.011	-1.007, 0.916	0.93
FOSL1	0.414	0.026, 0.186	**0.01***	0.503	0.046, 0.211	**0.003***
GSK3β	0.000	-0.281, 0.280	1.00	-0.105	-0.407, 0.175	0.43
c-JUN	0.069	-0.443, 0.775	0.59	0.035	-0.548, 0.714	0.79

802

c-MYC	0.078	-5.98, 9.69	0.63	0.013	-9.04, 9.64	0.95
SFRP1	0.025	-4.29, 5.00	0.88	0.156	-2.98, 7.34	0.90
SFRP2	0.088	-0.003, 0.005	0.50	0.113	-0.003, 0.006	0.44
WNT5A	0.111	-0.015, 0.038	0.39	0.072	-0.018, 0.033	0.56
WNT11	0.365	0.003, 0.021	**0.009***	0.472	0.005, 0.026	**0.006***

Data are presented as beta (β) coefficients and 95% confidence intervals (CIs). Model 1: unadjusted, Model 2: adjusted for age, gender, BMI, endoscopy procedure and smoking status. *P<0.05 for linear regression model.

806 *Table 4 Associations between the Dietary Inflammatory Index (E-DII) and rectal CCPS and*
807 *crypt dimensions at baseline*

808

	Model 1			Model 2		
Crypt measurement	**β coefficient**	**95% CI**	**P value**	**β coefficient**	**95% CI**	**P value**
Total mitoses	-0.055	-0.724, 0.457	0.65	-0.082	-0.853, 0.450	0.52
Proportion of mitotic cells in top half of the crypt	-0.036	-1.09, 0.802	0.77	-0.029	-1.17, 0.940	0.72
Length	-0.047	-8.60, 5.82	0.70	-0.084	-10.5, 5.50	0.59
Width	-0.074	-2.07, 1.10	0.54	-0.119	-2.55, 0.992	0.72
Volume	-0.096	-3.84×10^5, 2.15×10^5	0.57	-0.136	-4.37×10^5, 1.99×10^5	0.36

809 Data are presented as beta (β) coefficients and 95% confidence intervals (CIs). Model 1:
810 unadjusted, Model 2: adjusted for age, gender, BMI, endoscopy procedure and smoking
811 status. *P<0.05 for linear regression model.

FIGURE LEGENDS

Figure 1 Expression of WNT11 in the rectal mucosa of individuals with lower and higher E-DII scores at baseline.

Data are expressed as adjusted copies and presented as least square means following ANOVA GLM adjusted for age, gender, BMI, smoking status and endoscopy procedure. Error bars represent standard error of the mean. *p<0.05

Figure 2 Post-intervention expression of FOSL1 in the rectal mucosa of individuals with lower and higher E-DII scores given PD or placebo

Data are expressed as adjusted copies and presented as least square means following ANOVA GLM adjusted for age, gender, BMI, smoking status, endoscopy procedure and baseline (pre-intervention) expression.

Figure 1

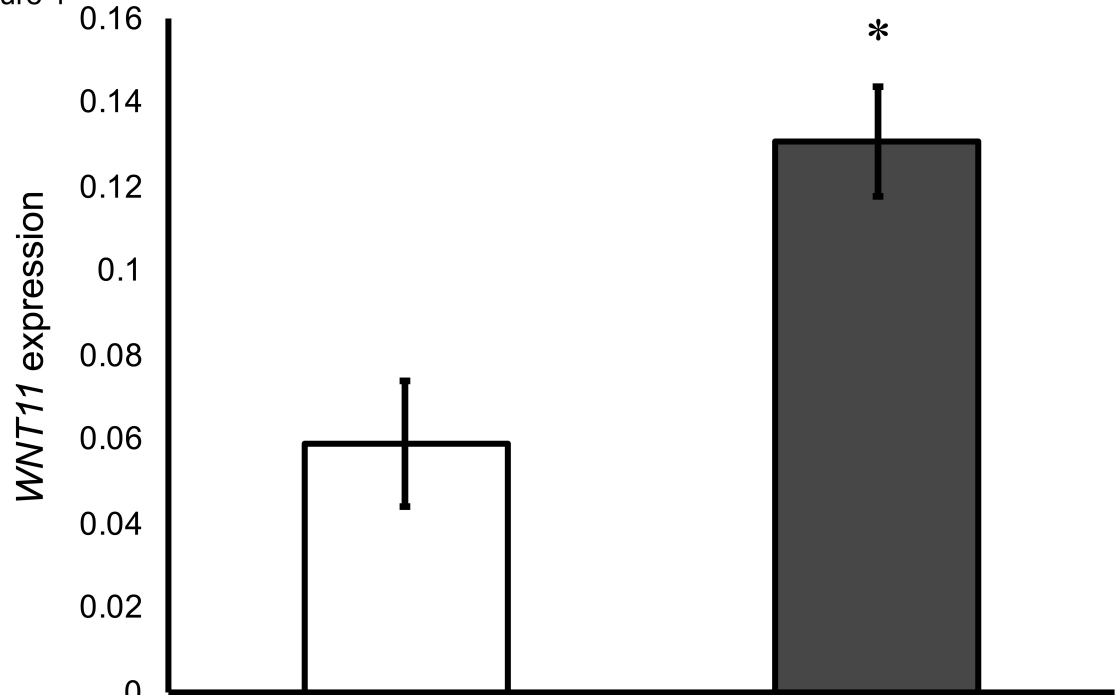
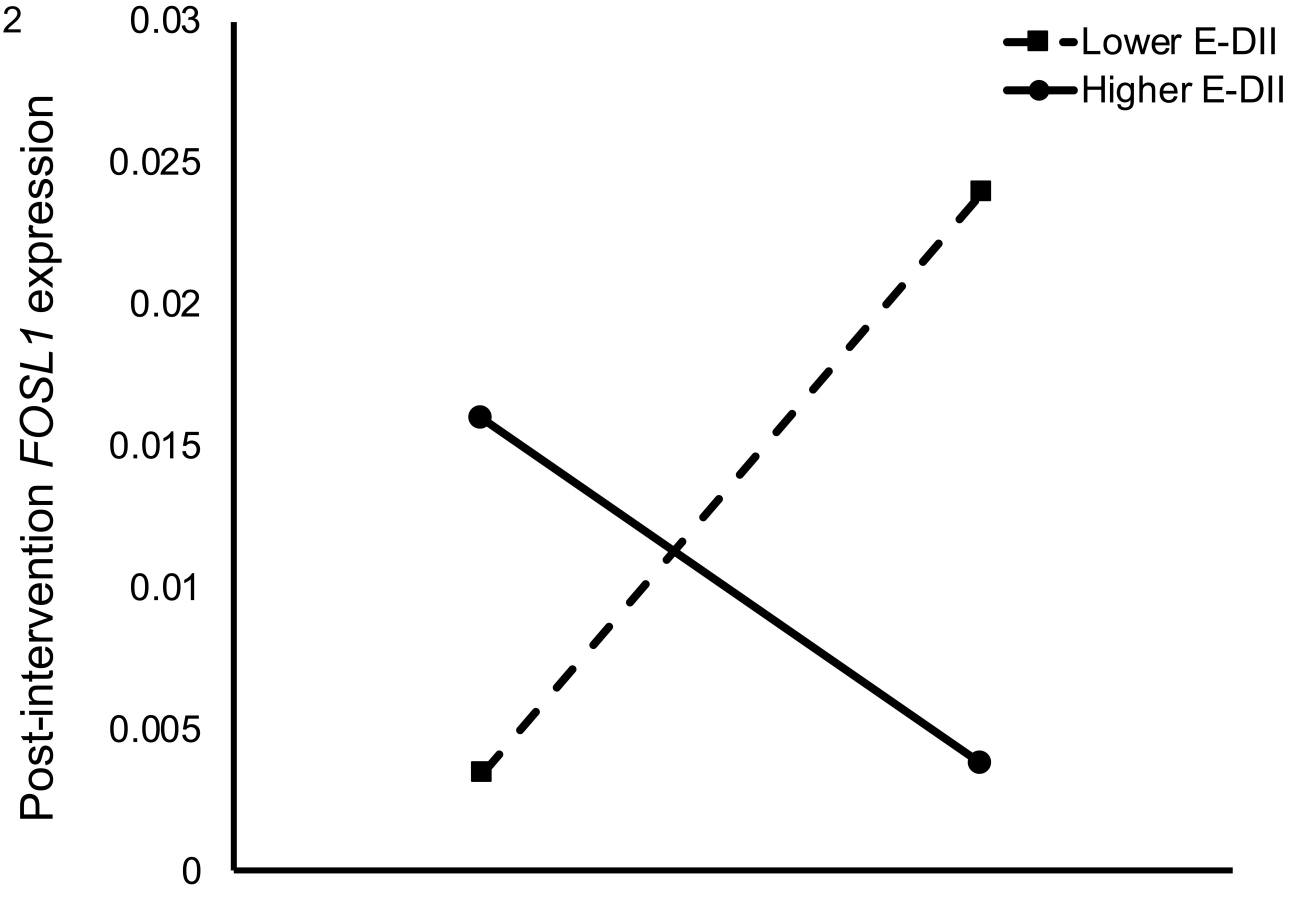


Figure 2



AACR American Association for Cancer Research

Cancer Prevention Research

Diet-associated inflammation modulates inflammation and WNT signaling in the rectal mucosa, and the response to supplementation with dietary fibre

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Cancer Prev Res Published OnlineFirst October 28, 2020.