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Is resistant starch protective against colorectal cancer via modulation of the WNT signalling pathway?

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Epidemiological and experimental evidence suggests that non-digestible carbohydrates (NDC) including resistant starch are protective against colorectal cancer. These anti-neoplastic effects are presumed to result from the production of the SCFA, butyrate, by colonic fermentation, which binds to the G-protein-coupled receptor GPR43 to regulate inflammation and other cancer-related processes. The WNT pathway is central to the maintenance of homeostasis within the large bowel through regulation of processes such as cell proliferation and migration and is frequently aberrantly hyperactivated in colorectal cancers. Abnormal WNT signalling can lead to irregular crypt cell proliferation that favours a hyperproliferative state. Butyrate has been shown to modulate the WNT pathway positively, affecting functional outcomes such as apoptosis and proliferation. Butyrate's ability to regulate gene expression results from epigenetic mechanisms, including its role as a histone deacetylase inhibitor and through modulating DNA methylation and the expression of microRNA. We conclude that genetic and epigenetic modulation of the WNT signalling pathway may be an important mechanism through which butyrate from fermentation of resistant starch and other NDC exert their chemoprotective effects.

Colorectal cancer: Non-digestible carbohydrates: Resistant starch: Butyrate: WNT signalling

Colorectal cancer (CRC) is the fourth most common cancer in the UK, and incidence has risen by 6% in the past 10 years⁽¹⁾. This cancer arises in colonocytes, the single layer of columnar epithelial cells which line the large bowel. In common with all cancer types, CRC develops because of genomic damage which gives the neoplastic cell new, and more competitive, characteristics⁽²⁾ enabling sustained proliferative signalling, evasion of growth suppressors, resistance to cell death, replicative immortality, the ability to induce angiogenesis and eventually, the potential for metastasis. While genetic susceptibility contributes to CRC risk, most CRC develop because of genomic damage caused by exogenous and endogenous processes and events. It has been estimated that over half of the CRC in the UK in 2010 resulted from environmental factors⁽³⁾ such as diet and physical activity and this provides support for the

hypothesis that a high proportion of CRC cases could be prevented through appropriate dietary and lifestyle choices or the development of suitable interventions. The World Cancer Research Fund /American Institute for Cancer Research Colorectal Cancer 2011 Report⁽⁴⁾ concluded that there is convincing evidence for increased risk of CRC with the consumption of red meat, processed meat and alcohol (in men) and also with increasing body and abdominal fatness. In addition, there is convincing evidence that more physical activity and the consumption of foods containing dietary fibre decreases CRC risk.

Dietary fibre and the modulation of CRC risk

Dietary fibre has been defined as carbohydrate polymers with ten or more monomeric units which are not

Abbreviations: ACF, aberrant crypt foci; CRC, colorectal cancer; DMH, dimethylhydrazine dihydrochloride; HAMS, high-amylose maize starch; NDC, non-digestible carbohydrates; RS, resistant starch; SFRP, secreted-frizzled related proteins.

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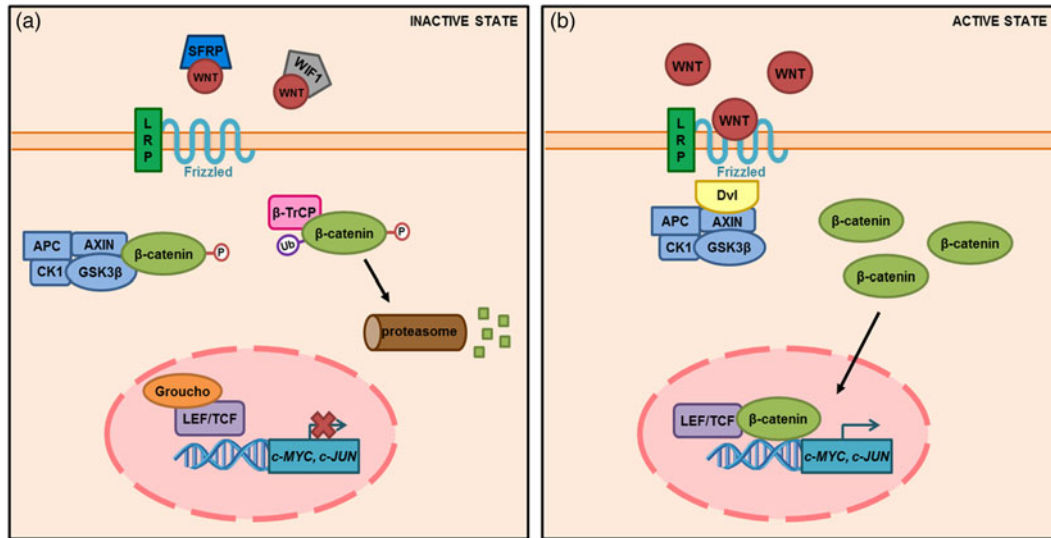


Fig. 1. The canonical WNT signalling pathway. (a) In the inactive state, when there are no WNT ligands bound to frizzled receptors or when WNT antagonists, such as WIF1, inhibit activation of the WNT pathway, the β -catenin destruction complex (adenomatous polyposis coli (APC), AXIN, casein kinase (CK1) and glycogen synthase kinase 3 β (GSK3 β)) binds to and phosphorylates β -catenin. This targets β -catenin for ubiquitination by β -transducin 117 repeat-containing protein (β -TrCP) and, consequently, proteasomal degradation. In the nucleus, lymphoid enhancer factor-1 (LEF) and T cell factor (TCF) transcription factors are repressed by Groucho. (b) When the WNT pathway is activated by binding of WNT ligands to frizzled receptors, the β -catenin destruction complex is inhibited by dishevelled (Dvl). Levels of dephosphorylated, active β -catenin rise and β -catenin translocates to the nucleus where it binds to LEF and TCF transcription factors to activate transcription of target genes such as *c-MYC* and *c-JUN*.

hydrolysed by the endogenous enzymes in the small intestine of human subjects⁽⁵⁾. It encompasses non-digestible carbohydrates (NDC) such as resistant starch (RS) and polydextrose. RS is the portion of dietary starch that avoids enzymatic digestion and absorption in the small intestine and consequently reaches the large bowel undigested⁽⁶⁾. Here, it is the target for fermentation by resident colonic bacteria to produce SCFA such as acetate, propionate and butyrate⁽⁷⁾. Four types of RS have been described: type 1 physically inaccessible RS, e.g. that are found in seeds; type 2 resistant granules, e.g. in uncooked potato or unripe banana; type 3 retrograded RS, found in cooked and cooled foods such as bread; type 4 chemically modified RS which is used widely for technological purposes in processed foods⁽⁷⁾.

Based on clinical observations in Africa, Burkitt was one of the first to report an association between higher consumption of dietary fibre and reduced CRC incidence⁽⁸⁾. Since then, many epidemiological studies have quantified links between dietary fibre intake and CRC risk. A recent meta-analysis of the results from twenty-five prospective studies confirmed an inverse correlation between dietary fibre intake and CRC risk⁽⁹⁾. The authors concluded that CRC risk is 10% lower for every 10 g/d increase in dietary fibre intake.

Early attempts to explain the protective effects of dietary fibre focused on the increased faecal bulk, reduced transit time and dilution of faecal content which were expected to reduce exposure of the large bowel to carcinogens^(10,11). More recently, the focus has shifted to the

beneficial effects of SCFA, in particular butyrate, which are produced by colonic fermentation of NDC⁽¹²⁾. Butyrate is produced predominantly by bacterial species of the *Clostridia*, *Roseburia* and *Eubacteria* genera⁽¹³⁾. Butyrate is the preferred energy source for colonocytes and plays an important role within the large bowel mucosa where it regulates key cellular processes including proliferation, differentiation and apoptosis that contribute to the maintenance of homeostasis⁽¹⁴⁾. Furthermore, butyrate has been reported to positively modulate WNT signalling⁽¹⁵⁾, a pathway that is frequently aberrantly activated in CRC, suggesting that this may be one of the mechanisms through which butyrate, and other NDC, are protective against CRC.

The WNT signalling pathway

WNT signalling comprises three different pathways: the canonical or β -catenin pathway, the non-canonical planar cell polarity pathway and the WNT/calcium pathway. The principal pathway, that is most relevant in human health and disease, is the canonical pathway. The canonical WNT signalling pathway is observed in several tissue and cell types⁽¹⁶⁾, including colonic crypts, where it regulates cell proliferation, migration and differentiation⁽¹⁷⁾.

In the inactive state, or when activation of the pathway is inhibited by antagonists such as secreted frizzled-related proteins (SFRP), the β -catenin destruction complex, comprising adenomatous polyposis coli, AXIN,

glycogen synthase kinase 3 β and casein kinase 1, phosphorylates β -catenin (Fig. 1a)^(17,18). Phosphorylated β -catenin is recognised by β -transducin repeat-containing protein which targets β -catenin for ubiquitination by the E3 ubiquitin ligase complex and, consequently, proteasomal degradation^(16,18). With low cytoplasmic concentrations of β -catenin, there is no translocation to the nucleus, transcription is not activated and the T cell factor and lymphoid enhancer factor-1 transcription factors are bound to, and inhibited by, Groucho and transducin-like enhancer of split. Activation of the canonical pathway occurs when a WNT ligand binds to a frizzled receptor, resulting in an interaction with co-receptors of the LDL receptor-related protein family (Fig. 1b). The β -catenin destruction complex is inactivated following inhibition of AXIN by dishevelled. Consequently, cytoplasmic levels of β -catenin rise and the protein is translocated to the nucleus where it binds to, and activates, T cell factor/lymphoid enhancer factor-1 transcription factors⁽¹⁹⁾, which switch on transcription of target genes such as *c-MYC* and *c-JUN*.

The WNT signalling pathway is also implicated in several cancers including CRC⁽²⁰⁾, where it is constitutively activated in approximately 90% of sporadic cases⁽²¹⁾. Aberrant expression of the WNT pathway results mainly from the loss of the *APC* tumour suppressor gene⁽²²⁾. However, genetic and epigenetic mechanisms also contribute to altered expression of other components of the pathway e.g. activating mutations in β -catenin⁽²³⁾ and down-regulation of *SFRP1* due to promoter hypermethylation⁽²⁴⁾ and, therefore, to tumour development.

The effects of resistant starch on colorectal carcinogenesis

Studies investigating the effects of RS on colorectal carcinogenesis have yielded inconsistent results. While some studies have indicated a chemoprotective effect of RS, others have reported no effect and some have observed adverse effects.

In rats treated with 1,2-dimethylhydrazine dihydrochloride (DMH; a carcinogen used widely in experimental studies of CRC formation), incidence of tumours was reduced when the animals were fed Novelose 330 (a type 3 RS) compared with those fed a standard diet⁽²⁵⁾. High-amylose maize starch (HAMS; a type 2 RS) has also been shown to reduce incidence and the number of adenocarcinomas in rats when the HAMS diet was provided for 4 weeks prior to injection with the carcinogen azoxymethane⁽²⁶⁾.

The formation of aberrant crypt foci (ACF), which are the earliest detectable pre-neoplastic lesions in CRC⁽²⁷⁾, is a marker of early-stage colorectal carcinogenesis and has been used as a surrogate endpoint in several studies investigating colorectal carcinogenesis in animal models. Using an azoxymethane-induced model of ACF, rats given raw potato starch (a type 2 RS) for 3 weeks post carcinogen treatment had significantly reduced ACF formation compared with controls⁽²⁸⁾. In contrast, rats fed moderate and high doses of RS prior to injection with

azoxymethane had a significantly greater number of ACF compared with those on the control diet and those on the low dose of RS. These results suggest that RS may be protective if given during the promotion stage of colorectal carcinogenesis i.e. following carcinogen treatment, but may have adverse effects at the pre-initiation stage⁽²⁸⁾. Consistent with findings from this study, two earlier animal studies reported protective effects of RS given during the promotion stage^(29,30). Thorup *et al.*⁽²⁹⁾ reported that there were significantly fewer ACF in azoxymethane-treated rats fed a potato starch-containing (RS) diet compared with those fed control or corn starch-containing diets. Similarly, rats fed RS (with or without a vitamin A supplement) for 12 weeks post-DMH injection had significantly reduced ACF formation⁽³⁰⁾.

Some studies have also reported no effect of RS on CRC in animal models. Although DMH-treated rats fed RS (3 or 10%) had significantly increased butyrate concentrations compared with those fed control diets, there were no effects of RS on the development of CRC⁽³¹⁾. Similarly, there was no effect on ACF formation in rats fed HAMS for 4 weeks prior to DMH injection⁽³²⁾.

In contrast, adverse effects of RS in promoting colorectal carcinogenesis have also been reported. Increased ACF density and colonic tumour formation were observed by Young *et al.* in DMH-injected rats fed potato starch for 31 weeks⁽³³⁾. It has been argued that carcinogen-treatment in animals may not be the best model for studies of human colorectal carcinogenesis and that genetic models which recapitulate aberrant WNT signalling because of *Apc* mutation may be more appropriate. Using the *Apc1638N* mouse model of sporadic CRC⁽³⁴⁾, we observed that feeding RS (a 1:1 blend of raw potato starch and Hylon VII, both type 2 RS) for 5 months increased tumour formation within the small intestine⁽³⁵⁾. However, this adverse effect was prevented with the synergistic administration of aspirin, which suggests that reducing inflammation may reduce CRC risk.

The effects of resistant starch and butyrate on the expression of WNT pathway components

The effects of RS on the expression of WNT pathway components have been investigated in only one published study in which carcinogen-treated rats were fed diets containing three corn maize varieties: an Argentinian strain, a Guatemalan strain and a hybrid of the two⁽³⁶⁾. Those animals fed the Argentinian strain, which yielded the lowest RS content, had enhanced β -catenin expression compared with those rats fed the hybrid diet. In addition, expression of the WNT antagonist, *SFRP4*, was significantly lower in rats fed the diets (Guatemalan strain and hybrid) with the highest RS content. Altogether, these results suggest that RS reduced WNT pathway activity in carcinogen-treated rats. However, this study also found that expression of two other negative regulators of WNT signalling, *AXIN2* and *WISPI*, was significantly reduced following the consumption of the two higher RS diets, which

would be indicative of reduced WNT pathway inhibition with RS. These observations underline the complexity of regulation of WNT signalling and the difficulty in drawing unequivocal conclusions.

In contrast with the paucity of studies with RS and WNT signalling, there are several studies, notably from the Bordonaro group, which have investigated the effects of butyrate on the expression of WNT pathway components. These investigators observed that treatment of SW620 colon carcinoma cells with 5 mM butyrate (a physiological concentration) induced apoptosis and that this was associated with increased activity of the T cell factor transcription factor indicating that the apoptotic effect may have been mediated through effects on WNT signalling⁽³⁷⁾. Furthermore, butyrate treatment increased formation of β -catenin–T cell factor complexes, which would activate transcription of target genes. A later study by the same group found that butyrate increased the levels of unphosphorylated, and therefore active, β -catenin following butyrate treatment in eight CRC cell lines⁽³⁸⁾.

Other studies have investigated effects of butyrate on another functional outcome of the WNT pathway i.e. differentiation. There is an evidence that butyrate treatment of LIM2537 colon cancer cells induces WNT signalling and that this is paralleled by greater differentiation of the, usually, poorly differentiated tumour cells⁽³⁹⁾. The activity of glycogen synthase kinase 3 β , a member of the β -catenin destruction complex, was significantly reduced in the butyrate-treated cells and, indeed, this led to stabilised pools of β -catenin within the cytoplasm which would be expected to increase WNT activity. There was an inverse correlation between glycogen synthase kinase 3 β expression and differentiation, suggesting that the induction of differentiation by butyrate resulted from an increase in WNT signalling. Unexpectedly, this was not paralleled by an increase in the expression of two target genes of the WNT pathway, *c-MYC* and *CCND1*.

Germann *et al.* observed that 4–5 mM butyrate treatment of CC531 rat colon carcinoma cells resulted in positive modulation of expression of four WNT pathway target genes (*CCND1*, *c-MYC*, *FOSL1* and *FST*), which have been reported to be up-regulated in CRC^(40–42). However, the effects of butyrate on *c-MYC* expression are complex. While increased *c-MYC* transcription is observed with butyrate-induced WNT signalling, this treatment may result in less *c-MYC* expression through inhibition of elongation causing a transcriptional block⁽⁴³⁾.

Studies using LT97 (a cell line representative of an early stage in the progression from the normal mucosa to adenomas) showed greater up-regulation of WNT signalling and, consequently, induction of apoptosis with butyrate treatment suggesting that early-stage neoplasms may be more responsive to the effects of butyrate⁽⁴⁴⁾.

A recent study by the Bordonaro group utilised total human microarray analyses to identify a total of 1587 direct and indirect targets of the WNT pathway whose expression was modulated by a physiologically-relevant concentration of butyrate (5 mM) in HCT-116 CRC cells⁽⁴⁵⁾. The differentially-expressed genes included those encoding

proteins that are involved in several key processes including differentiation, migration and DNA replication.

Modulation of WNT signalling by resistant starch and butyrate via epigenetic mechanisms

Gene expression may be regulated by epigenetic mechanisms, such as histone modifications, DNA methylation and the expression of microRNA (miRNA)⁽⁴⁶⁾. These mechanisms result in heritable changes in gene expression and function without alterations in the DNA sequence itself⁽⁴⁷⁾.

The role of butyrate as a histone deacetylase inhibitor

Histone acetylation refers to the addition of acetyl groups to the lysine residues on histone tails by histone acetyltransferase, removing the positive charge⁽⁴⁸⁾. Acetylated, relaxed DNA, known as euchromatin, is more easily accessible to transcription factors and may lead to increased transcription of the corresponding gene. On the contrary, histone deacetylation by histone deacetylase results in a more condensed DNA structure, known as heterochromatin, whereby transcription is reduced. Altered acetylation of histones associated with genes involved in regulation of the cell cycle, and particularly deacetylation of histone 4, has been linked with cancer development and progression⁽⁴⁹⁾.

The role of butyrate as a histone deacetylase inhibitor has been studied extensively and is well established⁽⁵⁰⁾ and butyrate treatment may restore expression of silenced genes leading to restoration of normal levels of proliferation, differentiation and apoptosis. Furthermore, butyrate affects acetylation of other non-histone targets, such as transcription factor Sp1⁽⁵¹⁾.

Resistant starch, butyrate and DNA methylation

DNA methylation describes the addition of a methyl group to the C₅ position of a cytosine residue that is followed by a guanine residue, known as a CpG site, resulting in 5-methylcytosine⁽⁵²⁾. DNA methylation is catalysed by a small family of DNA methyltransferases, primarily DNMT1, DNMT3a and DNMT3b⁽⁵³⁾. CpG islands refer to regions rich in CpG dinucleotides that are not normally methylated⁽⁵⁴⁾. Hypermethylation of CpG islands close to, or within, the promoter region is associated with repressed transcription principally through preventing transcription factor binding. DNA methylation can also inhibit transcription indirectly through steric hindrance by methyl-CpG-binding proteins which impede transcription factor binding^(54,55). In CRC and other cancers, global demethylation of DNA is observed frequently⁽⁵⁵⁾. In addition, promoter hypermethylation and, consequently, silencing of tumour suppressor genes is common in CRC as is hypomethylation and, therefore, up-regulation of oncogenes.

To date, there is only one published study of the effects of RS on DNA methylation of WNT pathway-related genes in a double-blind, randomised, placebo-controlled crossover trial with intervention periods lasting 4 weeks.

In this study, DNA methylation was quantified using *MethylLight*, a qPCR-based method, in rectal mucosal biopsies collected from seventeen healthy participants⁽⁵⁶⁾. Methylation of the promoter regions of sixteen genes, including a member of the WNT pathway, *SFRP1*, was quantified. *SFRP1* encodes a member of the SFRP family of WNT inhibitors and the down-regulation of *SFRP1*, associated with promoter methylation, is implicated in colorectal carcinogenesis⁽²⁴⁾. However, Worthley *et al.*⁽⁵⁶⁾ observed a significant ($P = 0.040$) effect of treatment on the methylation of *MINT2* only and concluded that this was likely due to chance.

There are no published studies of the effects of butyrate in CRC cells on the methylation state of members of the WNT pathway. However, in human gastric cancer cells, where, as in CRC, aberrant activation of WNT signalling is observed frequently, Shin *et al.*⁽⁵⁷⁾ showed that butyrate treatment restored *SFRP1* expression following promoter demethylation.

Resistant starch, butyrate and microRNA expression

miRNA are small, non-coding RNA that down-regulate the expression of their target genes by degrading mRNA or inhibiting translation. Approximately 1000 miRNA have been identified in human subject⁽⁵⁸⁾, with each single miRNA being able to target several genes and, likewise, a single gene may be targeted by many miRNA⁽⁵⁹⁾. Aberrant expression of miRNA, resulting in altered expression (usually down-regulation) of target genes involved in the regulation of proliferation, migration and differentiation, may contribute to carcinogenesis⁽⁶⁰⁾. Due to their recent discovery and the complexity in deciphering their many putative targets, investigations of the effects of NDC or butyrate on expression of miRNA that target genes from the WNT pathway specifically are limited.

A very recent randomised, controlled, crossover trial by Humphreys *et al.*⁽⁶¹⁾ was the first reported human study to investigate the effects of RS on miRNA expression. Twenty-three participants were randomised to either a high red meat diet or a high red meat diet plus butyrylated RS for 4 weeks. Red meat has been associated with increased CRC risk, and so the aim of the study was to examine whether RS could reverse detrimental effects of the high red meat diet, particularly altered expression of miRNA, in the colorectum. Following consumption of the high red meat diet, the investigators observed an increase of approximately 30% in expression of miRNA from the miR-17–92 cluster, an oncogenic cluster that is overexpressed in CRC. However, the effect was miRNAs specific and expression of five miRNA from this cluster, namely *miR-17*, *miR-19a*, *miR-19b*, *miR-20a* and *miR-92a* were significantly reduced in those fed butyrylated RS.

Microarray analysis of miRNA expression in HT29 colorectal adenocarcinoma cells treated with up to 10 mM sodium butyrate for 48 h revealed that a total of thirty-nine miRNA were up-regulated and thirty down-regulated⁽⁶²⁾. Subsequent validation by qPCR confirmed that expression of the selected miRNA belonging to the

miR-17–92 and miR-106a-363 clusters was reduced significantly following treatment with 5 mM butyrate.

The effects of resistant starch and butyrate on colonic crypt cell proliferation

Studies on the effects of RS and butyrate on crypt cell proliferation have yielded conflicting results. This divergence in findings is due, in part, to differences in the health status of the tissue under study. In some cases, mucosal cells from the normal colon respond to butyrate (and other SCFA) by increasing proliferation. In contrast, there is almost universal agreement that butyrate treatment of cancer cells suppresses proliferation⁽¹²⁾. Furthermore, differences in RS or butyrate dose, type of RS, participant/cell characteristics and length of treatment may influence responses and contribute to difficulties in drawing unambiguous conclusions from available data. For example, we have shown that the DNA mismatch repair status of cells determines their cell proliferative/apoptotic response to butyrate treatment⁽⁶³⁾.

Findings from studies that have investigated the effects of RS on colonic crypt cell proliferation are summarised in Table 1. The majority of the studies that have investigated the effects of RS supplementation on cell proliferation in the colon of healthy subjects have found no effect on proliferation. This includes the study by Wacker *et al.*⁽⁶⁴⁾ which administered the largest dose of RS (up to 59.7 g/d). In contrast, a number of studies have reported reduced cell proliferation in the colorectum of individuals with neoplasia^(25,26). Importantly, we have also shown that supplementation of CRC patients with a 1:1 blend of Novelose 240 and Novelose 330 (RS types 2 and 3) reduced the proportion of mitotic cells in the top half of the crypt⁽⁶⁵⁾. Study of the distribution of mitotic cells within the crypt (rather than measuring total proliferation within the whole crypt) may be a better indicator of CRC risk because alterations in the distribution of mitotic cells within the crypt have been observed to be one of the earliest detectable pre-malignant alterations in the apparently-normal mucosa of those at higher risk^(66,67).

Likewise, the findings from investigations of the effects of butyrate on colonic crypt cell proliferation are inconsistent. While there is an evidence that butyrate may increase cell proliferation in the healthy colon in specific circumstances^(68,69,70), based on our earlier observations in rats, we concluded that there is no convincing evidence that SCFA (or butyrate, specifically) are responsible for raising crypt cell proliferation above normal. In those instances where greater SCFA supply has been associated with increased crypt cell proliferation, the increase may be (1) from a hypoproliferative state towards a normal proliferative state, (2) a transient phenomenon accompanying tissue hypertrophy or (3) a homeostatic response to increased cell loss by cell sloughing or apoptosis⁽⁷¹⁾.

The differential responses of normal and cancer cells to butyrate treatment are referred to as the butyrate paradox. Comalada *et al.*⁽⁷²⁾ compared the effects of butyrate treatment on healthy fetal human colon cells and

Table 1. Studies that have investigated the effects of resistant starch (RS) on colonic crypt cell proliferation

Study	Subjects	Source of RS (g/d)	Duration (weeks)	Key findings
van Munster <i>et al.</i> ⁽⁹¹⁾	Fourteen healthy participants	45 g native amylo maize (28 g RS type 2)	2	Decreased cell proliferation
Wacker <i>et al.</i> ⁽⁶⁴⁾	Twelve healthy participants	Amylo maize (males 59.7 g and females 50.7 g RS type 2)	4	No effect on cell proliferation
Worthley <i>et al.</i> ⁽⁵⁶⁾	Twenty healthy participants	25 g HAMS (12.5 g RS)	4	No effect on cell proliferation
Burn <i>et al.</i> ⁽⁹²⁾	206 familial adenomatous polyposis patients	1:1 blend of potato starch and HAMS (30 g RS type 2)	1–12 years	No effect on cell proliferation
Dronamraju <i>et al.</i> ⁽⁶⁵⁾	Sixty-five CRC patients	30 g 1:1 blend of Novelose 240 (RS type 2) and Novelose 330 (RS type 3)	<4	Reduction in the proportion of mitotic cells in the top half of the crypt
van Gorkom <i>et al.</i> ⁽⁹³⁾	Eighty-six sporadic adenoma patients	30 g amylo maize (19 g RS type 2)	8	No effect on cell proliferation
Grubben <i>et al.</i> ⁽⁹⁴⁾	Twenty-three patients with recently removed adenomas	45 g amylo maize (28 g RS type 2)	4	No effect on cell proliferation
Bauer-Marinovic <i>et al.</i> ⁽²⁵⁾	Twenty male carcinogen-treated Sprague-Dawley rats	10 g RS type 3	20	Decreased cell proliferation
Clarke <i>et al.</i> ⁽⁸⁵⁾	Forty male carcinogen-treated Sprague-Dawley rats	HAMS and HAMSB	4	No effect on cell proliferation
Jacobasch <i>et al.</i> ⁽⁹⁵⁾	Rat model of ulcerative colitis	RS	2	Increased cell proliferation
Le Leu <i>et al.</i> ⁽²⁶⁾	Ninety male carcinogen-treated Sprague-Dawley rats	10 and 20/100 g HAMS (RS type 2)	4	Decreased cell proliferation
Winter <i>et al.</i> ⁽⁸⁶⁾	225 male c567bl/J mice	10/100 g HAMS (5/100 g RS type 2)	4 (short-term) and 18 months (long-term)	Increased cell proliferation with long-term supplementation
Mentschel and Claus ⁽⁹⁶⁾	Twelve male pigs	1.69 kg/d potato starch (RS type 2)	19 d	Increase in cell proliferation in middle and luminal compartments

HAMS, high amylose maize starch; HAMSB, butyrylated high amylose maize starch.

on HT-29 colorectal adenocarcinoma cells. Butyrate inhibited cell proliferation of the HT-29 cells but had no effect on the normal cells. Significantly reduced cell proliferation of HT-29 cells treated with 5 mM butyrate for 48 h has also been reported by Hodin *et al.*⁽⁷³⁾.

Bordonaro *et al.*⁽¹⁵⁾ have suggested that the differences in the effects of butyrate observed on proliferation in healthy compared with cancerous cells may be due to the sensitivity and responsiveness of these cells to butyrate. They proposed that, in cancerous cells where the WNT pathway is hyperactive, butyrate further induces WNT signalling and consequently apoptosis. However, in healthy cells where normal, moderate levels of WNT activity are found, butyrate contributes to the normal regulation of processes within the colon by the WNT pathway, such as the induction of proliferation. However, the latter is likely to increase only if starting from an abnormally low level⁽⁷¹⁾.

The effects of resistant starch and butyrate on apoptosis in the large bowel

A number of studies, mostly *in vivo*, have shown that increased WNT activity is associated with induction of apoptosis^(37,74,75). Furthermore, the Bordonaro group have reported a linear relationship between WNT activity and

levels of apoptosis in ten CRC cell lines⁽⁷⁶⁾. However, several *in vitro* studies have reported the opposite effect^(77–79).

In sixteen pigs supplemented with raw potato starch for 16 weeks, Nofrarias *et al.*⁽⁸⁰⁾ observed a significant reduction in apoptosis within the crypts of pigs fed the RS diet compared with controls. A similar earlier study also reported a reduction in apoptosis in the colon of pigs fed a potato starch diet and, interestingly, these researchers reported significantly higher faecal butyrate concentrations following the RS diet⁽⁸¹⁾.

The majority of studies have agreed that butyrate induces apoptosis⁽⁸²⁾, primarily via induction of the intrinsic and extrinsic apoptotic pathways. Furthermore, butyrate has been reported to modulate the expression of apoptotic genes including down-regulation of the anti-apoptotic gene *BCL-2*⁽⁸³⁾ and up-regulation of the pro-apoptotic gene *BAX*⁽⁸⁴⁾. *In vivo*, increased apoptosis has been observed in both the distal and proximal colon of carcinogen-treated rats fed a diet with RS type 3⁽²⁵⁾. Furthermore, significantly greater levels of apoptosis have been found in the distal colon of carcinogen-treated rats fed a butyrylated high amylose maize starch, which produces significantly greater concentrations of butyrate in the colon, compared with HAMS and a low RS diet⁽⁸⁵⁾. In addition, this increase in apoptosis correlated positively with distal colonic luminal butyrate concentrations, suggesting that the enhanced apoptosis was a

consequence of the extra butyrate production. However, some studies have reported no effect of butyrate on levels of apoptosis in the colon^(86,87).

Conclusions

The WNT signalling pathway is central to normal function of the colorectal epithelium and aberrant WNT signalling is a cardinal feature of most CRC. Since high intakes of NDC are associated with lower CRC risk, this review investigated the evidence that NDC such as RS may have this protective effect through impacts on WNT signalling. Such effects may be mediated by butyrate, a major SCFA endproduct of RS fermentation in the large bowel. Several studies have observed positive modulation of WNT pathway components by RS and by butyrate and, in some cases, these have correlated with protective effects on functional outcomes such as apoptosis and differentiation. The effects of RS and butyrate on the expression of WNT pathway-related genes may result from epigenetic mechanisms including inhibition of histone deacetylation, reduction of DNA methylation and altered expression of miRNA. In particular, butyrate reduces the methylation state of *SFRP1*, which is frequently silenced in CRC as a consequence of hypermethylation, in cancer cells. In addition, RS and butyrate reduce expression of miRNA from the oncogenic miR-17-92 cluster both *in vitro* and *in vivo*. Numerous studies have reported effects of RS and its fermentation product, butyrate, on cell proliferation and apoptosis, which may be regulated by the WNT pathway. However, the effects of both RS and butyrate on proliferation and apoptosis appear to differ markedly between normal and tumour cells. In the healthy crypt, RS and butyrate contribute to the maintenance of homeostasis by WNT signalling through promoting (initially low) levels of proliferation and by reducing apoptosis. However, in cancerous cells, where the crypt is in a hyperproliferative state and has high levels of WNT signalling, RS and butyrate reduce proliferation and induce apoptosis. It must be noted, however, that the effects of RS and butyrate on these two processes may not be due exclusively to effects on WNT signalling and that modulation of additional pathways including the Notch and MAPK signalling pathways is also likely to be important^(88,89). To confirm the chemoprotective effects of RS and butyrate, and to better understand the mechanisms through which these effects are mediated, well-designed, randomised, placebo-controlled dietary intervention studies are required. In the only such human randomised controlled trial study to date with cancer as the endpoint, there was no evidence that supplemental RS affected the development of CRC⁽⁹⁰⁾ and we concluded that dietary supplementation with RS does not emulate the apparently protective effects of diets rich in dietary fibre against CRC.

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Conflicts of Interest

None.

Authorship

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