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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Relationships between adherence to the Mediterranean diet and methylation of WNT pathway-related genes implicated in colorectal cancer

Bo Geurts
Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :
dr. Sabine LANGIE

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Prof. John C. MATHERS

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Dr. Fiona MALCOMSON

Transnational University Limburg is a unique collaboration of two universities in two countries: the University of Hasselt and Maastricht University.

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List of abbreviations

ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
BFU	BORICC Follow-up
BORICC	Biomarkers Of Risk in CRC
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
DNMT	DNA methyltransferases
EVOO	Extra virgin olive oil
FFQ	Food Frequency Questionnaire
GLM	General linear model
hMLH1	Human mutL homolog 1
LINE-1	Long interspersed nuclear element-1
MD	Mediterranean diet
MDS	Mediterranean diet score
MMR	Mismatch repair
MSI	Microsatellite instability
MUFA	Monounsaturated fatty acids
PREDIMED	PREvención con DIeta MEDiterránea
WIF1	WNT inhibitory factor

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Abstract

Introduction: Colorectal cancer (CRC) is the 3rd most common cancer in the world and is strongly influenced by environmental factors including diet. Adherence to a Mediterranean dietary pattern is associated with a lower incidence of all-cause mortality and an inverse association with CRC risk has been reported. WNT signalling is a pathway involved in maintaining a healthy large bowel but which is hyperactive in up to 85% of sporadic CRC because of abnormal methylation and expression of WNT pathway genes. Altered WNT signalling is a potential mechanism for the effects of diet on CRC risk.

Objective: To investigate relationships between adherence to a Mediterranean diet (MD) and methylation of WNT pathway genes implicated in CRC.

Methods: A literature search identified three genes (*WNT5A, WNT6* and *WNT10A*) associated with MD and CRC. DNA was extracted from rectal biopsies of 40 BFU Study participants, bisulphite-modified, and PCR-amplified to identify DNA methylation at specific CpG sites by pyrosequencing. The MD score (MDS) was calculated from habitual dietary intake data (max. score 14).

Results: In this study 40 participants were included (age range 49-79 years, 52.5% female). MDS ranged from 2-7 and participants were divided into low- and high-MDS groups by dichotomising at the median (4.5). Mean rectal *WNT5A*, *WNT6*, and *WNT10A* methylation was relatively low across all participants i.e. 8.04%, 1.82% and 2.66%, respectively. There were no significant relationships between adherence to the MD and methylation of *WNT5A* or *WNT10A* (P>0.05). However, CpG site 5 within *WNT6* was positively correlated with MDS (P=0.016). Mean methylation of all three genes did not differ between participants with lower and higher MDS (P>0.05). However, a *post hoc* analysis showed that *WNT5A* methylation was lower in participants with polyps compared with healthy participants. For participants with polyps, methylation of *WNT5A* was almost 50% lower in those with lower MDS compared to those with higher MDS (P=0.032), however statistical significance was lost after adjusting for covariates (P>0.05).

Conclusion: No evidence was found to support the hypothesis that MD adherence is associated with *WNT10A* or *WNT5A* methylation. However, *WNT6* methylation at CpG5 correlated with MDS. In addition, for polyp participants only, differences in *WNT5A* were observed between those with lower and higher MDS. Further research is needed to elucidate the relationship between WNT pathway genes and adherence to a MD, especially in those at higher CRC risk.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer in the world and the fourth most common cause of cancer-related death (1). It affects around 1.36 million people worldwide every year, and causes approximately 694 000 deaths annually (2). CRC is especially common in economically developed countries, where an estimated 60% of all cases occur (3). The survival rate of patients who are diagnosed early is 50% at 5 years. However, when patients are diagnosed at a later metastatic stage of CRC survival rate at 5 years is only approximately 12% (4). The risk of developing CRC increases with advancing age. More than 90% of persons diagnosed with CRC are older than 50, with an average age at diagnosis of 64 years (5). CRC is strongly influenced by environmental factors including diet. It has been estimated that nutrition could account for more than one third of cancer deaths (6), and that dietary factors are responsible for 70% to 90% of all cases (7). In the past few decades, findings from extensive epidemiological and experimental investigations have linked consumption of several foods and nutrients, such as dietary fibre and red meat, to the risk of colorectal neoplasia. Therefore, dietary modifications could serve as a preventative measure to reduce the global burden of CRC (8). A Western diet is characterised by higher consumption of red and processed meat and reduced intake of dietary fibre which are associated with higher CRC risk. In contrast, people living along the Mediterranean coast who eat a traditional Mediterranean-style diet, which primarily includes plant based food, have decreased overall cancer mortality (3).

CRC is a 'silent' disease that takes years or even decades to develop. It usually arises sporadically and is mostly asymptomatic until progression to an advanced stage. CRC develops through a multistage process that includes epigenetic, as well as genetic, alterations in a number of oncogenes and tumor suppressor genes including DNA mismatch repair (MMR) genes, and cell cycle regulating genes in colonic mucosal cells (4, 9, 10). These genetic and epigenetic changes result in three major molecular pathways i.e. chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP) through which CRC develops (4).

MSI develops through the defects in the MMR system either via epigenetic silencing of *hMLH1* in CpG islands by promoter hypermethylation or a point mutation of the MMR genes (*MLH1, MSH2, MSH6,* and *PMS2*). These alterations of the DNA drive the progression of CRC. Around 10–15% of CRCs are caused by defective DNA MMR machinery associated with *MLH1* hypermethylation (4, 11, 12). MSI is a molecular hallmark of hereditary non-polyposis CRC,

also known as the Lynch syndrome, which is caused by a germ-line mutation in one of the MMR genes.

The CIN pathway is characterized by widespread imbalances in chromosome number (aneuploidy) and loss of heterozygosity (13). In the CIN pathway, adenomatous polyposis coli (*APC)* mutations are the most common. APC is a multifunctional protein with an important role in the WNT pathway and the regulation of processes including cell cycle control, cytoskeleton stabilization, intracellular adhesion and apoptosis. Familial adenomatous polyposis, a major hereditary predisposition event leading to CRC development, results from germ-line mutations in the *APC* gene. *APC* mutations are found in more than 80% of sporadic colorectal tumors (14, 15).

The third molecular pathway in the aetiology of CRC is the CIMP pathway. CpG islands are genomic regions with a high frequency of CpG dinucleotides and are often associated with the transcription start sites of genes (16). CpG islands within the promoter regions are normally unmethylated and methylation of these CpG islands silences the activity of the corresponding genes (17). CIMP occurs in multiple types of cancer; these cancer cells often have a loss of global DNA methylation and a gain of methylation at the promoters of specific CpG islands. As a result, hundreds of non-mutated genes per cancer cell are silenced, some of which are tumor suppressor genes. Whilst these changes in patterns of DNA methylation are characteristic of CIMP cancers, there are no universally accepted criteria to define a tumor as CIMP-high. It has been estimated that CIMP-high occurs in about 20% of sporadic CRCs (13).

1.1 DNA methylation in colorectal cancer

As noted above, CRC develops from a multistage process as a result of both epigenetic and genetic alterations. Epigenetic alterations are mitotically heritable changes to the DNA which do not affect the underlying DNA sequence but can influence its function. The three inter-dependent mechanisms involved in epigenetics are DNA methylation, histone modification and non-coding microRNAs. These three mechanisms interact closely to regulate chromatin structure and gene expression (16).

DNA methylation is the enzymatic addition of a methyl group to the 5-position of a cytosine residue by DNA methyltransferases (DNMT) to produce 5-methylcytosine. DNA methylation is a normal mechanism in the mammalian genome by which cells regulate gene expression, and the majority of the CpG sites in the human genome, which are located outside of promoter

regions, are heavily methylated. For example, DNA methylation is a major mechanism for X chromosome inactivation and genomic imprinting (18). In mammals, the majority of DNA methylation marks occur on the 5' position of cytosine residues, where the cytosine is followed by guanine residue in the 5' to 3' direction, i.e. a so-called CpG dinucleotide that are distributed widely over the genome (19). Although the majority of cytosine residues within CpG dinucleotides are methylated, some, particularly those in CpG islands within the promoter regions, are normally unmethylated. Methylation of CpG islands occurs in many genes that are expressed in colonic epithelial cells (17). Global (whole genome) DNA methylation is 10-40% lower in CRCs compared with normal colonic tissue, primarily due to the loss of methylation within the repetitive elements such as long interspersed nuclear element-1 (*LINE-1*) and *ALU* (20). It is thought that this contributes to CRC initiation by enhancing genomic instability (21). Additionally, CRCs are characterized by hypermethylation of a subset of gene promotors (20), in particular hypermethylation of cytosine residues in the CpG islands of tumor suppressor genes (22).

1.2 WNT signalling pathway

The WNT signalling pathway plays a critical role in normal intestinal function and in maintaining the health of the large bowel. Dysregulation of the WNT pathway is a crucial step in CRC tumorigenesis, which is characterized by the accumulation of both genetic and epigenetic changes (23). *APC* inactivation by mutation is the most common WNT pathway alteration and results in the constitutive activation of canonical WNT signalling in CRC (explained in Figure 1) (24). Abnormal WNT pathway activity can result from aberrant methylation of WNT pathway components, particularly WNT inhibitors and ligands, resulting in abnormal expression of these genes. Promoter hypermethylation of negative regulators of the canonical WNT pathway, such as secreted frizzled-related proteins, dickkopf family proteins and *WIF1* (WNT inhibitory factor), has also been described in CRC (24).

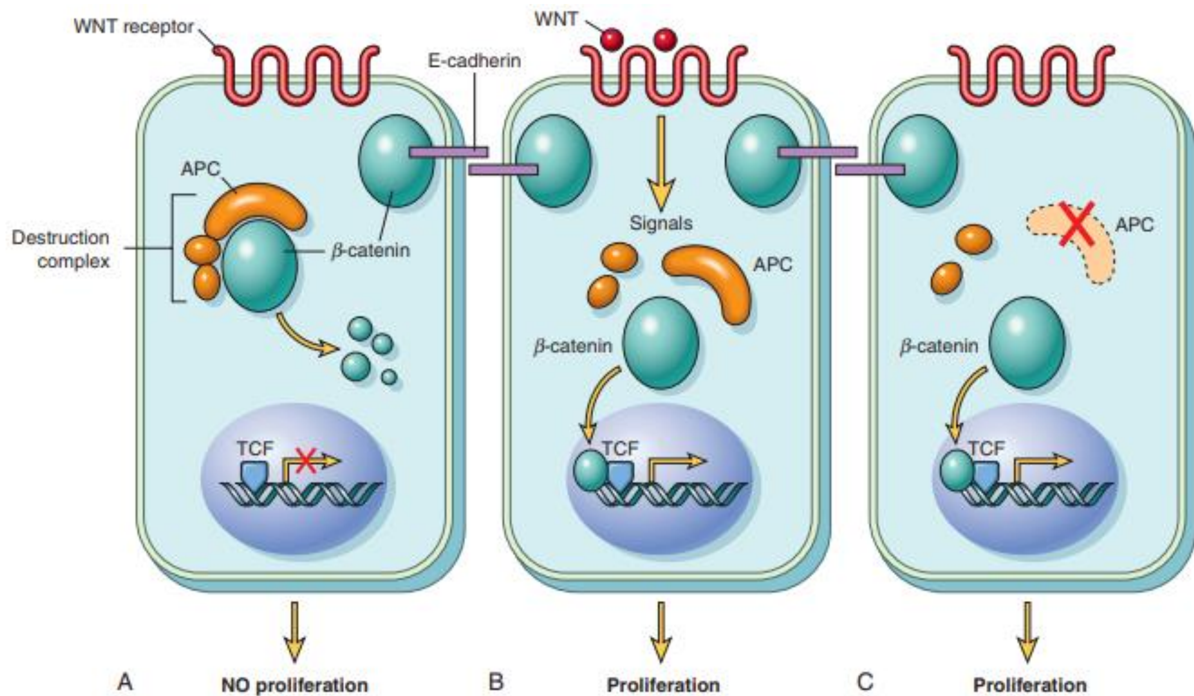

Figure 1: WNT signalling pathway. A) In resting cells that are not exposed to WNT, β-catenin forms a complex containing the APC protein. This complex ensures the destruction of β-catenin. Intracellular levels of β-catenin are low. B) When cells are stimulated with WNT molecules, the destruction complex is deactivated, and β-catenin degradation does not occur. The cytoplasmic levels of β-catenin increase, and β-catenin translocates to the nucleus where it binds to TCF. TCF is a transcription factor that activates several genes involved in the cell cycle. C) In the case that APC is mutated or absent, the destruction complex of β-catenin cannot occur. B-catenin translocates to the nucleus and coactivates genes that promote the cell cycle. The cells behave if they are under constant stimulation by WNT (11, 25-27).

1.3 Risk factors for colorectal cancer

Both genetic and acquired or environmental factors can increase the risk of CRC development. Despite the greater impact of genetic susceptibility in an individual compared with environmental factors, the vast majority of CRC could be prevented through modifications in environmental factors. In the past few decades, diet has been a popular subject of CRC research because of its potential as both a risk factor and a protective factor (28). High intakes of red and processed meats and low intake of dietary fibre increase CRC risk (29-31), and unhealthy diets increase the risk of CRC by up to 70% (9).

1.3.1 Mechanisms trough which diet may alter colorectal risk

The mechanisms through which nutrition modifies CRC risk include effects on DNA methylation. There are at least two mechanisms through which nutrients may affect DNA methylation (32). Firstly, nutrients may influence the supply of methyl groups for the synthesis of S-adenosylmethionine (SAM) and, therefore, the biochemical pathways of methylation (33).

For example, folate is a one-carbon donor which provides methyl groups for the synthesis of nucleic acid purine bases and for DNA methylation (34). Epidemiological data have suggested that higher intakes of folate and higher folate status are associated with lower risk of CRC (35, 36), but this has not been confirmed in folate intervention studies (32). The second mechanism is associated with the fact that nutrients may alter the activity of enzymes such as DNMT (33). For example, a number of polyphenols found in plant foods are competitive inhibitors of DNMT1 and can result in reversal of hypermethylation of tumor suppressor genes in cancer cells (37). In addition, in a study of mice fed a high-fat diet, fish oil supplementation abolished the decrease in *Pparg2* promoter methylation in skeletal muscle, thereby suppressing the increase in *Pparg2* expression (34, 38).

1.4 Mediterranean diet

The Mediterranean diet (MD) is known to be one of the healthiest dietary patterns. It is associated with a lower incidence of all-cause mortality (39, 40) as well as with reduced risk of cardiovascular diseases (41), type 2 diabetes (42), certain types of cancer (43) and neurodegenerative diseases (39, 44). Di Francesco *et al.* provided *in vivo* and *in vitro* evidence that supplementation with extra virgin olive oil (EVOO) decreased DNA methylation of *CB1* – a tumor suppressor associated with a cancer phenotype (45). In addition, *CB1* expression was correlated inversely with DNA methylation at the *CNR1* promoter and was associated with reduced proliferation of Caco-2 cells (a well-studied colon cancer cell line). EVOO represents the typical lipid source of the MD (45) and these authors suggested that modulation of *CB1* expression by EVOO or its phenolic compounds via altered DNA methylation may represent an anti-cancer mechanism of this key component of the MD. Traditionally, the MD pattern (Figure 2) is a plant-based diet in which vegetables, fruit, cereals (preferably whole grain), legumes and nuts are consumed in a high amount and frequency. This dietary pattern also includes moderate consumption of fish and shellfish, white meat, eggs, and dairy products. On the contrary, the MD contains low consumption of red meat, processed meats and foods rich in sugars and in fats in both quantity and frequency. The beneficial effects of this dietary pattern on cardiovascular disease risk may be due its beneficial fatty acid profile, with a high content of monounsaturated fatty acids (MUFA) and a higher MUFA/saturated fatty acids ratio in contrast with the non-MDs. However, the high consumption of dietary fibre, low glycemic index and glycemic load, anti-inflammatory effects and antioxidant compounds present in the foods contributing to the MD may act together to produce the beneficial effects on cancer risk and on other health outcomes. Additionally there are also qualitative cultural and lifestyle

elements that are part of the MD such as conviviality, culinary activities, physical activity and adequate rest that may be important for health and well-being (46).

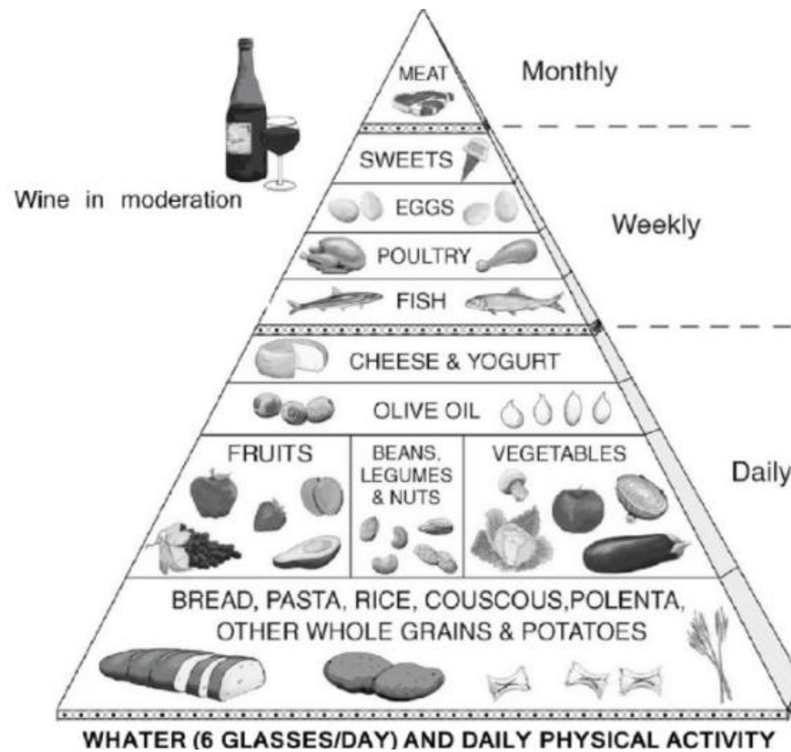

Figure 2: Mediterranean diet pyramid (47)

1.5 Mediterranean diet and colorectal cancer risk

Several studies have investigated the relationships between adherence to a MD and the risk of developing CRC. A systematic review and a meta-analysis confirmed an important inverse association between adherence to the MD and overall cancer mortality (RR_{cohort}: 0.86, 95% CI 0.81 to 0.91, $I^2 = 82\%$; n = 14 studies) and showed lower risk of several cancer types, especially colorectal cancer ($RR_{observational}$: 0.82, 95% CI 0.75 to 0.88, $I^2 = 73\%$; n = 11 studies) (31). The beneficial effects observed in these studies appeared to be driven mainly by higher intakes of fruits, vegetables and whole grains (31). One study with a total of 465 colorectal cancer cases found that women adhering to a MD pattern had lower CRC risk (48). In the multivariable adjusted model, the test for trend was positive (HR = 0.88, 95% CI: 0.78 to 0.99; Ptrend = 0.03) for a 2-point increment in the Mediterranean diet score. As noted above (section 1.3.1), evidence is growing that the effect of nutrition on CRC risk may be mediated by epigenetic mechanisms (49). However, further research is needed to determine whether the protection against CRC by the MD occurs through epigenetic mechanisms which modulate the expression of genes commonly de-regulated in CRC.

1.6 Hypothesis and objectives

1.6.1 Hypothesis

In this project we hypothesize that the methylation of WNT pathway genes is modulated by adherence to the MD and differs between individuals with lower and higher MD score.

1.6.2 Aim

This project aimed to test this hypothesis by investigating the link between the adherence to the MD and the methylation of CRC-related genes in the colorectal mucosa. In a secondary analysis, we investigated i) whether participants with a prior history of adenomatous polyps (and who, therefore, were at higher CRC risk) had differential methylation levels and ii) whether there is differential methylation in healthy vs polyp participants in relation to adherence to a MD.

1.6.3 Objectives

- Perform a literature search and select genes for which expression and/or methylation is associated with MD and with CRC.
- Extract DNA from rectal mucosal biopsies from participants in the BFU Study.
- Bisulphite-modify extracted DNA.
- Amplify selected target genes by PCR.
- Quantify DNA methylation at specific CpG sites within selected genes by pyrosequencing.
- Statistically analyse relationships between MD and methylation of selected genes.
- Undertake secondary statistical analysis to determine whether participants with a prior history of adenomatous polyps had differential methylation levels.
- Further explore this difference to investigate whether effects of adherence to the MD on DNA methylation differed in healthy vs polyp participants.

2. Methods

2.1 The BFU Study

This study was part of the BORICC (Biomarkers Of Risk in CRC) Follow-Up (BFU) Study. The BORICC Study recruited 363 participants in 2005/6 with the aim of investigating relationships between lifestyle factors, such as diet, and markers of CRC risk (17). The BORICC Study included two arms: the first recruited 262 healthy participants and the second recruited 101 participants with a prior history of adenomatous polyp, and therefore at higher risk of CRC. The BFU Study is a 12+ year longitudinal study which aims to enhance the understanding of the relationships between ageing, diet, physical activity and health of the bowel. Volunteer recruitment and sample collection took place between March 2017 and June 2018 at the North Tyneside General Hospital (Tyne and Wear, UK). Ethical approval for the BFU Study was obtained from the West Midlands – Coventry & Warwickshire Research Ethics Committee on 29th November 2016 (REC No. 16/WM/0424). Caldicott approval for the storage of data was provided by the Northumbria NHS Foundation Trust. The BFU Study is conducted in accordance with the declaration of Helsinki. Participants from the original BORICC study were contacted again for re-recruitment and informed consent was obtained from those volunteers who wished to participate in the BFU Study. All participants recruited to the BORICC Study in 2005/6 were invited to take part in the BFU Study, and exclusion criteria included being unable to provide informed written consent (e.g. participants who had developed dementia) and anyone actively undergoing chemotherapy or radiotherapy. Individuals on anticoagulant or anti-platelet medication were invited to take part in all parts of the study except the collection of colorectal biopsies due to greater risk of bleeding. The BFU Study participants included both healthy individuals and those who had adenomatous polyps removed at baseline in the BORICC Study. The following samples were collected from the volunteers: colorectal mucosal biopsies, blood, urine, stool and buccal swabs. All the biological samples were stored at -80 degrees until use. In addition, the volunteers were subjected to extensive phenotyping including the collection of data on habitual diet, physical activity, smoking behaviour, anthropometry and medical history.

2.2 Mediterranean diet assessment

Habitual diet was assessed by asking participants to complete a food frequency questionnaire (FFQ) to estimate the frequency of food consumption over the previous 12 months. Raw data from the FFQ were processed according to the criteria originally used in the PREDIMED study (50) to calculate a total MD score (MDS). Because of the limited detail of foods consumed as

reported in the FFQ, a number of assumptions were made for certain foods when calculating the MDS for individual participants. Where possible these assumptions were made based on detailed criteria devised by Shannon *et al.* (In press) (51). Table 1 summarises the operationalisation of the MD scoring system used in this project. The scoring system included 14 dietary components, for example 'using olive oil as opposed to other culinary fats'. If the participant adhered to this recommendation, or consumed the recommended serving size, one point was allocated per component. The total MDS was calculated from the sum of the score for each component, with a maximum score of 14. NB dietary analyses to provide MDS for each BFU Study participant was carried out by other members of the Mathers Laboratory (52).

Table 1: Operationalisation of the MD scoring system

Component	MD Scoring system	Serving size
1	Olive oil more than other culinary fat	-
2	Olive oil (≥4 tbsp/d)	11g
3	Vegetables (≥2 servings/d)	80g
4	Fresh fruits (including natural fruit juice; ≥3 servings/d)	80g for fresh or 150 ml for juice
5	Red processed meats (<1 serving/d)	150g
6	Spread fats (butter, magarine, cream; <1 serving/d)	12g
7	Soda drinks (<1 drink/d)	250ml
8	Wine with meals (Only for habitual drinkers; ≥7 glasses/wk)	175ml
9	Legumes (≥3 servings/wk)	150g
10	Fish (especially fatty fish), seafood (≥ servings/wk)	150g
11	Commercial bakery goods, sweets, and pastries (<3 servings/wk)	60g
12	Tree nuts and peanuts (≥3 servings/wk)	30g
13	White meat instead of red meat	-
14	Sofrito (sauce made with tomato and onion, leek, or garlic, simmered with olive oil; ≥2 servings/wk)	-

2.3 Target gene selection strategy

A literature search was performed to identify potential target genes for the DNA methylation analysis (Figure 3). A review of the literature covering full-text English articles in PubMed was conducted until December 2018. First, a literature search was done to find genes that are modulated by the MD through expression or methylation (Figure 3; Step 1). In the second step, identified genes were immediately selected if the studies were performed in colorectal tissue or

cells (Step 2.1). If this was not the case, an additional search was performed to identify genes where expression or methylation levels have been reported in colorectal tissue or cells (Step 2.2). In the third step, identified genes were immediately selected if they were related to CRC (Step 3.1). If this was not the case, an additional search was performed to identify if the genes are linked with CRC (Step 3.2). Lastly, only genes that were aberrantly methylated in CRC were selected (Step 4). Because most of these genes belonged to the WNT signalling pathway, only the genes belonging to this pathway were selected (Step 5). The results of this literature search can be found in section 3.1.

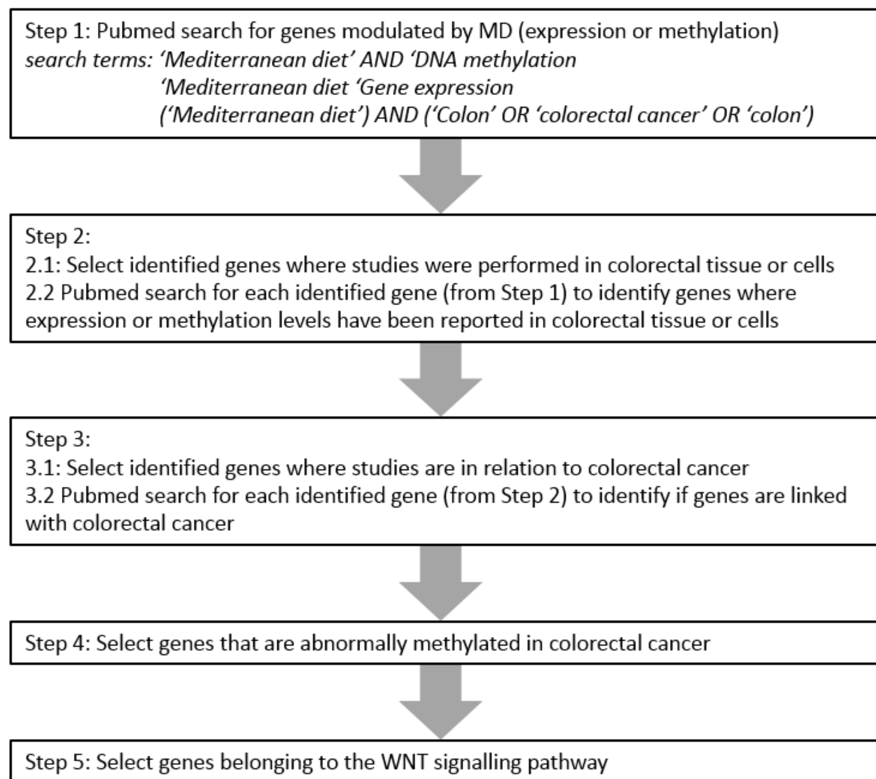

Figure 3: Methods gene selection

2.4 DNA extraction from rectal biopsies

DNA extraction was performed using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, USA). Rectal biopsies were transferred to tubes containing 180µl of lysis solution T and 20µl Proteinase K solution, and the mixtures were vortexed. The samples were incubated at 55°C in a shaking heat block at 800rpm for 2-4 hours, with occasional vortexing for complete enzymatic digestion. Next, 200µl lysis solution C were added to the samples before vortexing for 15 seconds, followed by a 10-minute incubation at 70°C. 20µl RNase solution A were added to degrade the RNA and the samples were incubated at room temperature for 2 minutes. To maximize DNA binding to the membrane, which results in more consistent

yields, 500µl of column preparation solution were added to the pre-assembled columns and centrifuged at 12000g for 1 minute. The flow-through was discarded. 200µl of 99.8% ethanol were added to the sample and vortexed to homogenise the solution. Next the lysate was transferred to the treated binding column, which was centrifuged at 6500g for 1 minute before discarding and replacing the collection tube. 500µl of wash solution were added to the column and centrifuged as. Again, 500µl of washing solution were added to the column, the flow-through was discarded after centrifugation at 14000g for 3 minutes. A fourth centrifugation step was performed at 14000g for 1 minute to ensure all solutions were removed. Next, the binding column was placed in a new collection tube. To elute the DNA from the column, 50µl of elution solution were added into the centre of the column and centrifuged at 6500g for 1 minute after an incubation of 5 minutes at room temperature. The yield of the DNA (ng/µl) and the purity ratios (A260/280 and A260/230) were assessed using the Nanodrop 2000 (Thermofisher scientific, USA) spectrophotometer (Supplement 1 and 2).

2.5 Bisulphite modification of the DNA

The EZ DNA methylation-gold^{TM} kit (Zymo research, USA) was used to bisulphite-modify the extracted DNA. First, the CT conversion reagent was prepared by adding 900 µl water, 300 µl of M-dilution buffer, and 50 µl of M-dissolving buffer to a tube of CT conversion reagent. This was mixed at room temperature with frequent vortexing or shaking for 10 minutes. The M-wash buffer was prepared by adding 24 ml of 100% ethanol to 6 ml M-Wash Buffer concentrate (D5005) before use. For each DNA sample, 130 µl of the CT conversion reagent and 20 µl DNA sample were added to a PCR tube. The PCR tubes were placed in a thermal cycler (Bio-Rad Laboratories Inc, USA); these steps are described in Supplement 2.

After the Thermal cycler steps were complete, 600 µl M-binding buffer were then added to a Zymo-Spin^{TM} IC column and the column placed into a collection tube. The sample was loaded into the spin column and mixed by inverting the column several times. This was centrifuged at full speed (>10,000 x g) for 30 seconds and the flow through was discarded. 100 µl of M-wash buffer were added to the column and centrifuged at full speed for 30 seconds. Next, 200 µl of M-desulphonation buffer were added to the column. This was incubated at room temperature for 15-20 minutes. Afterwards, the column was centrifuged for 30 seconds at full speed. Then, 200 µl of M-wash buffer were added to the column and centrifuged at full speed for 30 seconds. The washing step was repeated one more time. Lastly, the column was placed into a 1.5 ml microcentrifuge tube and 10 µl M-elution buffer was directly added to the column matrix. The DNA was eluted from the column by centrifuging for 30 seconds at full speed.

2.6 Primers for PCR amplification and pyrosequencing

Commercially available Pyromark CpG assays (Qiagen, Germany) were used for the amplification of bisulphite modified DNA by PCR and pyrosequencing (Table 2).

Table 2: Pyromark CpG primers

Assay	Chromosomal location	Primer (bp)	Target sequence	CpG sites
WNT5A	Chr3, BP 555151099-55515318	219	TCGCTGGCGTGCCCCGC GCACAGGATCCCAGCGA	5
WNT6	Chr2, BP 219724784-219725047	263	GTCCGCCGACGTGCGCC GGGC	5
WNT10A	Chr2, BP 219745021-219745091	70	GTCTGTGCGCGCGT	3

2.7 Amplification of bisulphite-modified DNA by PCR

Amplification of bisulphite-modified DNA was performed using the PyroMark® PCR kit (Qiagen, Germany). For each PCR, a master mix was prepared by scaling up the volumes in Table 3 for the number of reactions required. 23µl of master mix were pipetted into 0.2ml PCR tubes, followed by 0.5µl of bisulphite-modified rectal DNA sample. In addition, a negative control was included with water replacing the rectal DNA.

Table 3: PCR master mix components

PCR reaction mix component	Volume per reaction (µl)
GoTaq® Hot Start Green Master Mix, 2X	12
Nuclease-free water	9
PCR primer	2

All PCR reactions were performed using a thermal cycler (Bio-Rad Laboratories Inc, USA) under set conditions (Table 4). Before the amplification of the samples, the annealing temperature for each primer was tested (Supplement 3). Agarose gel electrophoresis was used to confirm PCR product and absence of contamination in the negative control sample (Supplement 4).

Table 4: Thermal cycler steps for amplification by PCR

Step	Temperature (°C)	Duration (minutes)	Number of cycles
Initial denaturation	94	2	1
3-step cycling:			40
Denaturation	94	0.40	
Annealing	50.4	0.40	
Extension	72	40	
Final extension	72	5	1

2.8 Quantification of DNA methylation by pyrosequencing

Pyrosequencing is a quantitative method that monitors the real-time incorporation of nucleotides. It is based on sequencing-by-synthesis and on the detection of released pyrophosphate that is converted by enzymes into a proportional light signal during DNA synthesis. The pyromark Q96 ID pyrosequencer and Pyromark Gold Q96 reagents (Qiagen, Germany) were used to perform this analysis.

In a 96-well PCR plate, 70 µl of the binding buffer solution were pipetted into each well. The binding buffer solution consists of beads, binding buffer and mili-Q H_2O (Table 5). 10 µl of PCR product were added to each well. The PCR plate was sealed and shaken at room temperature at 1400 rpm for 10 minutes.

Table 5: Master mix

	Per well (µl)
1 x binding buffer	40
Streptavidin-sepharose beads	2
PCR product	10
Mili-Q H_2O	28

While the PCR plate was shaking, the annealing buffer solution was prepared. The annealing buffer solution consists of 38.4 µl annealing buffer and 1.6 µl sequencing primer (10 pM). 40 µl of the annealing buffer solution were added into each well of the Pyromark Q96 plate. Using the Qiagen PyroMark Q96 Vacuum Workstation to aspirate the samples from the PCR plate, the filter probes were there flushed with 70% ethanol for 5 seconds, followed by denaturation solution for 5 seconds and wash buffer for 10 seconds. The vacuum pump was then switched off and the beads were released by shaking the probes gently from side to side in the

pyrosequencing low plate. The plate was heated at 80°C for 2 minutes and then cooled to room temperature for 10 minutes. The nucleotides, enzyme and substrate solutions were added into the designated compartments of the reagent cartridge. The cartridge and plate were inserted, and the assay was run on the pyrosequencer. The pyrosequencer has an internal control to assure the quality of the bisulphite modified DNA.

2.9 Data processing

Pyrosequencing provides quantitative estimates of DNA methylation (%) at specific CpG sites on amplified, bisulphite modified DNA. Non-concordant (not within 5% methylation) or 'failed' duplicates determined by the PyroMark software were repeated. If repeated, the two most concordant percentages from the two sets of duplicates were selected for statistical analysis, providing the data were within 5% concordance. If 5% concordance was not achieved after repeating these samples, the average of all 4 readings was calculated for subsequent analyses.

2.10 Statistical analysis

Statistical analyses were carried out using Minitab 17 Statistical Analysis Software. Before analysis, the Kolmogorov-Smirnov test was applied to datasets to assess whether data were normally-distributed ($P>0.05$). Correlation analyses were performed to investigate relationships between total MDS and methylation levels, and to investigate the correlation of methylation at CpG sites within a gene. Pearson's correlation was performed for normally-distributed data. Data that were not normally distributed were analysed with Spearman's Rho correlation. Prior to running categorical statistical analyses, participants were organised into two categories based on their MDS by dichotomising at the median (MDS=4); participants scoring 2 to 4 points were allocated to the "Lower MDS group" and participants scoring 5 to 7 points were allocated to the "Higher MDS group" (scores ranged from 2 to 7). Categorical analyses were performed to investigate differences in methylation between those with lower and higher MD adherence. The Mann-Whitney test was used when the data were not-normally distributed. For normally distributed data, the t-test was used. The analysis of variance (ANOVA) general linear model (GLM) was used to analyse the difference in methylation of the target genes between the MDS groups, adjusting for age, gender, BMI and smoking status as covariates. An interaction analyses between health status (healthy participants and participants with polyps) and MDS category was performed (data not shown). A p-value of <0.05 was considered statistically significant for all statistical analyses performed. .

3. Results

3.1 BFU Study participant characteristics

Table 6 gives an overview of the characteristics of the 40 BFU Study participants for whom rectal mucosal biopsies were collected. 31 participants were from the healthy group, and 9 had a prior history of polyps (polyp groups). The mean (10th-90th percentile) age of the participants was 66.4 (55.8-78) and ranged from 49 to 79 years. The percentage of females in the BFU study was 52.5%. The mean BMI of the participants was 27.98 kg/m2 and the mean body fat was 34.31%. The participants mean weight and waist circumference were 81.40 kg and 95.83 cm, respectively. Most of the participants had never smoked (52.5%), 45% were former smokers and 2.5% smoked daily. Most of the participants consumed alcohol 1 to 2 times per week (35%). While 25% of participants reported consuming alcohol almost daily, 15% and 25% reported consuming alcohol 1 to 2 times per month or occasionally, respectively, and 5% reported no consumption of alcohol. There was only one participant who consumed 2 or more alcoholic drinks per day.

Table 6: BFU Study Participant characteristics (n=40)

Characteristic	n (%) or mean (10th – 90th percentile)
Age (years)	66.43 (55.8-78)
Female	21 (52.5)
Healthy	31 (77.5)
BMI (kg/m2)	27.98 (22.4-34.3)
Body fat (%)	34.31 (24.36-44.11)
Weight (kg)	81.40 (59.98-104.24)
Waist circumference (cm)	95.83 (75.49-112.94)
Smoking status	
Never smoker	21 (52.5)
Former smoker	18 (45)
Daily smoker	1 (2.5)
Alcohol consumption	
None	2 (5)
Occasionally	7 (17.5)
1-2 per week	14 (35)
1-2 per month	6 (15)
Almost daily	10 (25)
2 a day or more	1 (2.5)

3.2 Mediterranean diet score

The total MDS ranged from 2 to 7, with a possible maximum score of 14 (Figure 4). The most common scores for the BFU participants were 4 and 5 and the mean MDS was 4.5. The participants were divided into two categories based on their MDS by dichotomising at the median (MDS=4.5), with 20 participants (50%) in the lower MDS category and 20 participants (50%) in the higher MDS category.

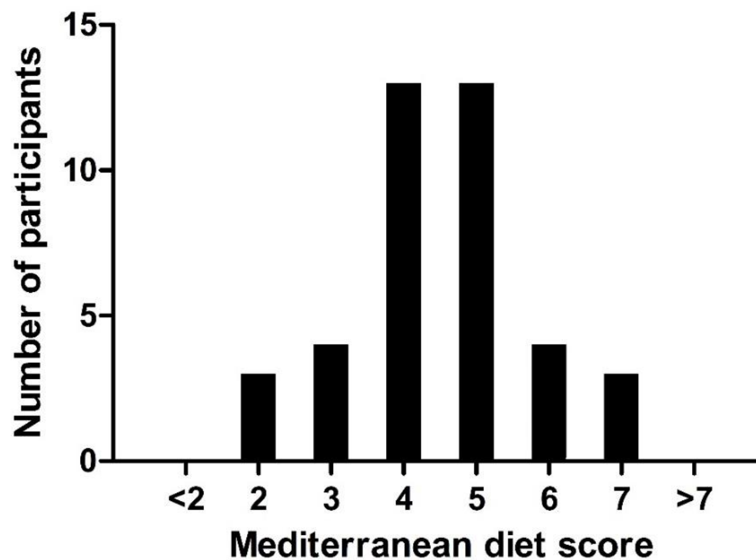

Figure 4: Distribution of total MDS for BFU Study participants (n=40)

3.3 Gene selection

After the first literature search, a total of 29 genes were found whose methylation or expression levels were modulated by the MD (Figure 5, Step 1). From these 29 genes, 24 genes were identified where expression or methylation levels have been reported in colorectal tissue or cells (Step 2). Results of the final search revealed that 21 genes are implicated in colorectal carcinogenesis (Step 3). From the identified 9 genes that are abnormally methylated in CRC risk (Step 4), 4 genes were identified that are part of the WNT signalling pathway: *WNT5A, WNT6, WNT8 and WNT10A* (Step 5). The availability of the PyroMark® CpG primer assays for the target genes was checked on Gene Globe (Qiagen website). As primers were available for *WNT5A, WNT6,* and *WNT10A* only, these three genes were selected for investigation in this project.

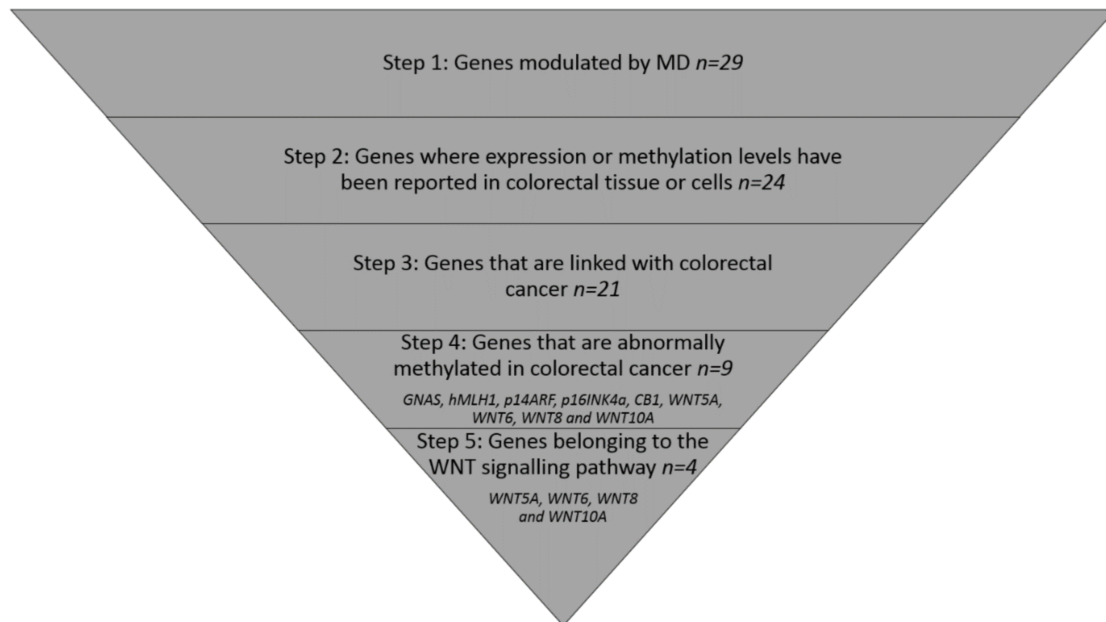

Figure 5: Results of gene selection process

<u>*3.4 WNT5A methylation*</u>

Methylation of *WNT5A* was quantified at 5 CpG sites. Mean methylation across all CpG sites in all participants was 8.04% (±2.06). The highest methylation was 10.94% (±2.86) at CpG site 5, and the lowest was 4.08% (±3.00) at CpG site 4 (Figure 6).

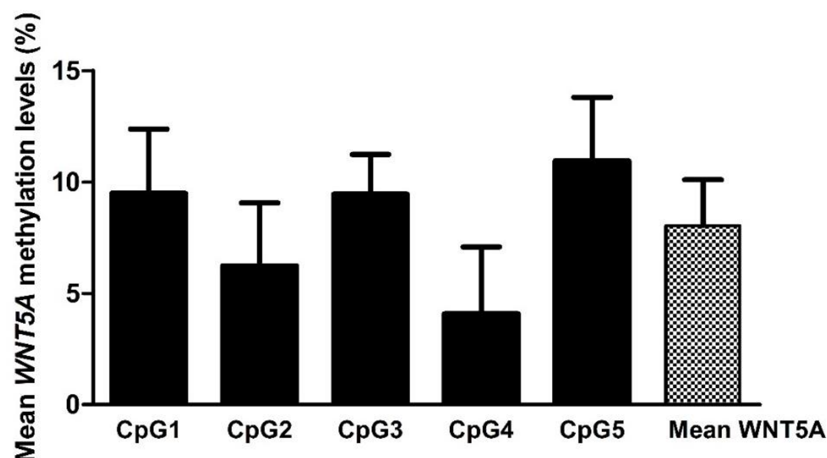

Figure 6: Mean WNT5A methylation at all CpG sites.

3.4.1 Correlations between WNT5A methylation at all analysed CpG sites

The correlation coefficients of the analyses between methylation at each CpG site in *WNT5A* ranged from 0.351 to 0.608. All the correlations between different CpG sites were positive and statistically significant ($p<0.05$) (Figure 7).

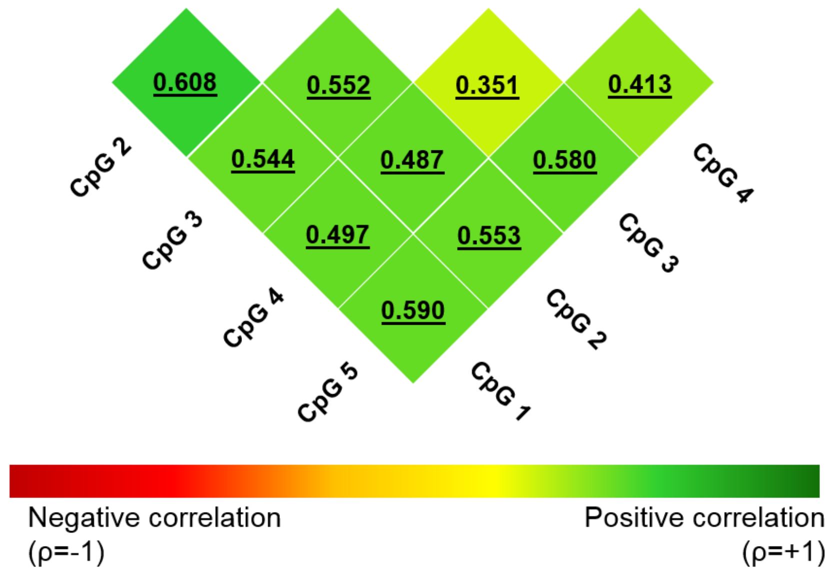

Figure 7: Heat map showing correlations between methylation at individual CpG sites within WNT5A. Spearman's correlations were performed. Statistically significant correlations (p<0.05) are underlined and in bold (p-values not shown).

3.4.2 Relationships between MD score and methylation of WNT5A

Relationships between the MDS and *WNT5A* methylation were investigated using correlation analyses. No statistically significant relationships were observed between MDS and mean methylation across all CpG sites (Figure in Supplement 6) or methylation at any CpG site (p>0.05) (Table 7).

Table 7: Correlations between the MD score and methylation of specific sites within WNT5A.

***WNT5A* methylation**	**Pearson correlation coefficient**	**P-value**
CpG site1	0.131	0.419
CpG site2[a]	0.035	0.831
CpG site 3	-0.098	0.548
CpG site 4[a]	0.100	0.541
CpG site 5	-0.025	0.878
Total methylation	0.111	0.494

[a] *Spearman Rho correlation coefficient*

3.4.3 Differences in WNT5A methylation between lower and higher MDS groups

Differences in *WNT5A* methylation levels between lower and higher MDS groups were investigated for all five CpG sites and total methylation using the Mann-Whitney test for the non-normally distributed data and the t-test for the normally distributed data (Table 8). There were no significant differences between lower (mean 8.1%) and higher (mean 8.01%) MD adherers for total *WNT5A* methylation or methylation at each of the five CpG sites (P>0.05).

Further, the effects of age, gender and BMI on *WNT5A* methylation modulation by MDS category as covariates were investigated using the ANOVA GLM; no statistically significant effects of age, gender or BMI were observed (P>0.05).

Table 8: Difference between and methylation of specific CpG sites within WNT5A

***WNT5A* methylation**	**Lower MDS (0-4) Mean (SEM or SD) (n=20)**	**Higher MDS (5-14) Mean (SEM or SD) (n=20)**	**Unadjusted P-value[1]**	**Adjusted P- value[2]**
Total methylation	8.072 (0.482)	8.010 (0.482)	0.706	0.817
CpG site1	9.627 (0.658)	9.357 (0.658)	0.730	0.780
CpG site2[a]	3.742	6.723	0.607	/
CpG site 3	9.520 (0.389)	9.379 (0.389)	0.748	0.804
CpG site 4[a]	3.742	5.290	0.695	/
CpG site 5	11.199 (0.659)	10.690 (0.659)	0.669	0.599

[a]*Data are presented as medians, Mann-Whitney.*
[1]*T-test*
[2]*ANOVA GLM adjusted for age, gender, BMI, and smoking status.*
Only for normally distributed data, the ANOVA GLM was calculated.

3.5 WNT6 methylation

Methylation of *WNT6* was quantified at 5 CpG sites. Mean methylation across all CpG sites in all participants was 1.82% (±1.87). The highest methylation was 4.01% (±3.53) at CpG site 1, and the lowest was 0.07% (±0.46) at CpG site 3 (Figure 8).

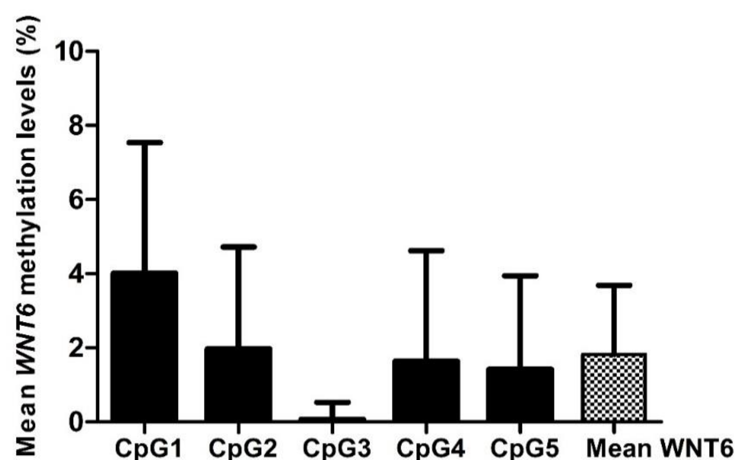

Figure 8: Mean WNT6 methylation at all CpG sites.

3.5.1 Correlations between WNT6 methylation at all analysed CpG sites

The correlation coefficients of the analyses between methylation at each CpG site in *WNT6* ranged from 0.255 to 0.480 (Figure 9). All of the correlations between different CpG sites were

positively correlated. Whereof 7 out of 10 correlations were statistically significant (p<0.05). The strongest correlation can be seen between CpG site 5 and 4 with a correlation coefficient of 0.480 (P=0.002).

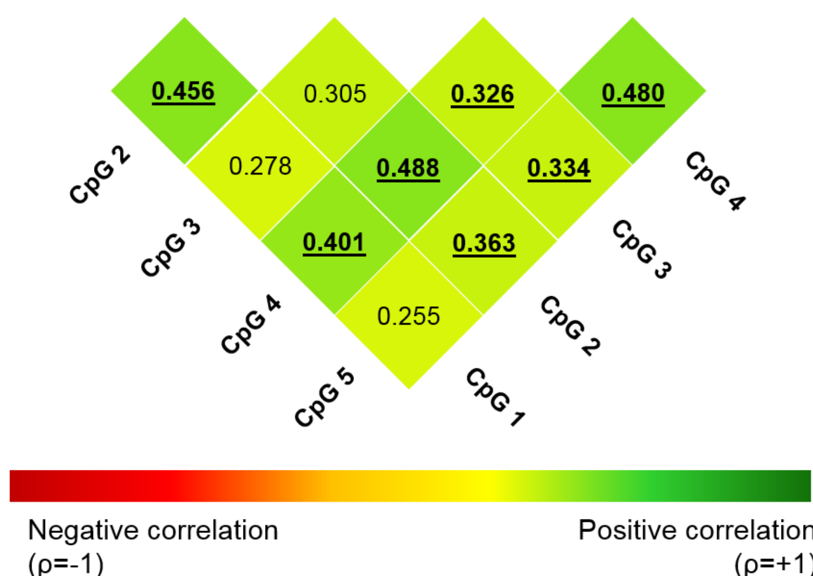

Figure 9: Heat map showing correlations between methylation at individual CpG sites within WNT6. Spearman's correlations were performed. Statistically significant correlations (p<0.05) are underlined and in bold (p-values not shown).

3.5.2 Relationship between MD score and methylation of WNT6

Relationships between the MDS and *WNT6* methylation were investigated using correlation analyses. A statistically significant positive correlation was found between MDS and methylation at CpG site 5 (correlation coefficient= 0.378, P=0.016) (Figure 10). No statistically significant relationships were observed between MDS and mean methylation across all CpG sites (Figure supplement 6) or methylation for the other CpG sites (Table 9) (p>0.05). However, there was a trend for a positive correlation between MDS and methylation at CpG site 3 (P=0.097).

Table 9: Correlations between the MD score and methylation of specific CpG sites within WNT6.

***WNT6* methylation**	**Spearman Rho correlation coefficient**	**P-value**
CpG site1	0.184	0.257
CpG site2	0.143	0.378
CpG site 3	0.266	0.097
CpG site 4	0.121	0.459
CpG site 5	0.378	**0.016**
Total methylation	0.160	0.324

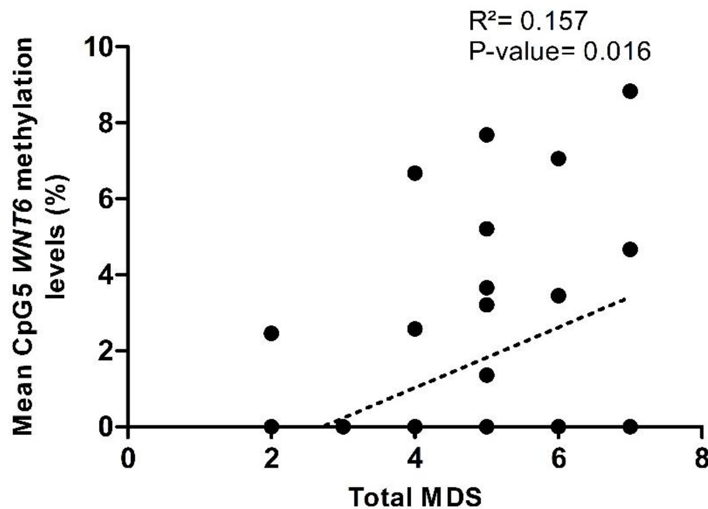

Figure 10: Correlation between MD score and CpG site 5 WNT6 methylation. Spearman Rho correlation coefficient 0.378; P-value= 0.016.

3.5.3 Differences in WNT6 methylation between lower and higher MDS groups

Differences in *WNT6* methylation levels between lower and higher MDS groups were investigated for all five CpG sites and total methylation using the Mann-Whitney test for the non-normally distributed (Table 10). There were no significant differences between lower (median 1.11%) and higher (median 1.77%) MD adherers for total *WNT6* methylation or methylation at each of the five CpG sites (P>0.05). However, there was a significant trend for CpG site 5. Methylation at CpG site 5 appeared to be lower in participants with a lower MDS (mean methylation 0.59%) compared with those with a higher MDS (mean methylation 2.26%) (P=0.08). Further, the effects of age, gender and BMI on *WNT6* methylation as covariates were investigated using the ANOVA GLM and no statistically significant effects were observed (P>0.05).

Table 10: Difference between total MDS and methylation of specific CpG sites within WNT6.

***WNT6* methylation**	**Lower MDS (0-4) Mean (SEM or SD) (n=20)**	**Higher MDS (5-14) Mean (SEM or SD) (n=20)**	**Unadjusted P-value[1]**	**Adjusted P-value[2]**
Total methylation[a]	1.113	1.765	0.1167	/
CpG site1[a]	2.320	5.457	0.1136	/
CpG site2[a]	0	0	0.2674	/
CpG site 3[c]	/	/	/	/
CpG site 4[a]	0	0	0.4249	/
CpG site 5[b]	0.59	2.26	0.0787	/

[a] Data is presented as medians, Mann-Whitney.
[b]Mann-Whitney test performed, but data presented as means.
[c]Mann-Whitney test could not be performed.
[1]T-test
[2]ANOVA GLM adjusted for age, gender, BMI, and smoking status.
Only for normally distributed data, the ANOVA GLM was performed.

3.6 WNT10A methylation

Methylation of *WNT10A* was quantified at 3 CpG sites. Mean methylation across all CpG sites in all participants was 2.66% (±1.11). The highest methylation was 4.27% (±1.51) at CpG site 1, and the lowest was 1.60% (±1.98) at CpG site 2 (Figure 11).

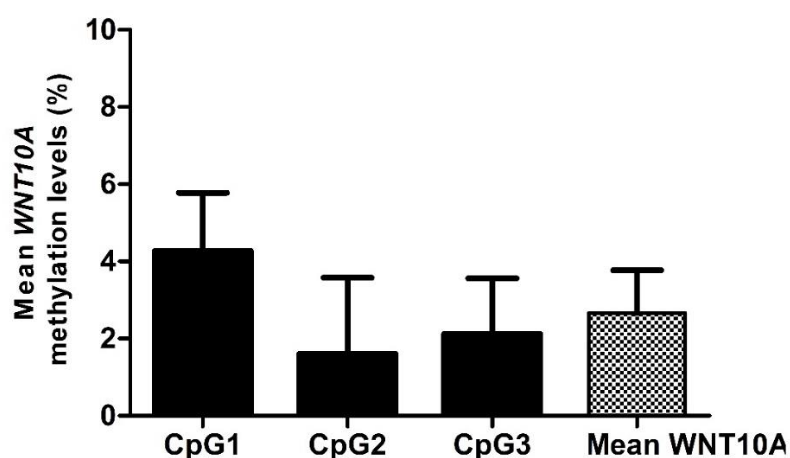

Figure 11: Mean WNT10A methylation at all CpG sites.

3.6.1 Correlations between WNT10A methylation at all analysed CpG sites

The correlation coefficients of the analyses between methylation at each CpG site in *WNT10A* ranged from -0.269 to 0.527 (Figure 12). Only the correlation between CpG site 2 and 3 was negative. A statistically significant, positive correlation was observed between methylation at CpG site 1 and 2 ($p<0.001$).

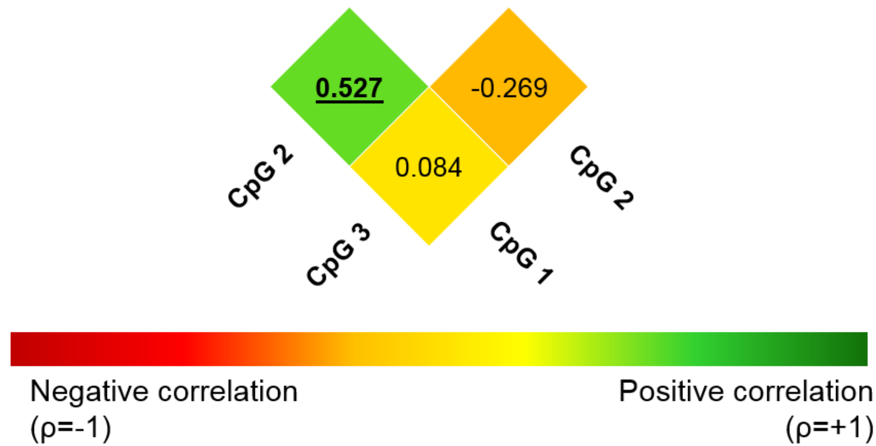

Figure 12: Heat map showing correlations between methylation at individual CpG sites within WNT10A. Pearson's correlations were performed. Statistically significant correlations (p<0.05) are underlined and in bold (p-values not shown).

3.6.2 Relationship between MD score and methylation of WNT10A

Relationships between the MDS and *WNT10A* methylation were investigated using correlation analyses. No statistically significant relationships were observed between MDS and mean methylation across all CpG sites (Figure in Supplement 6) or methylation at any CpG site (p>0.05) (Table 11).

Table 11: Correlations between the MD score and methylation of specific CpG sites within WNT10A.

***WNT10A* methylation**	**Spearman Rho correlation coefficient**	**P-value**
CpG site1	0.043	0.794
CpG site2	0.091	0.575
CpG site 3	-0.074	0.650
Total methylation[a]	0.126	0.437

[a] *Pearson's correlation coefficient*

3.6.3 Differences in WNT10A methylation between lower and higher MDS groups

The difference in *WNT10A* methylation between lower and higher MDS groups was investigated for all the three CpG sites (Table 12) using the Mann-Whitney test. A t-test was used to investigate the difference in total *WNT10A* methylation between lower and higher MDS groups. There were no significant differences between lower (mean 2.2%) and higher (mean 2.1%) MD adherers for total *WNT10A* methylation or methylation at each of the three CpG sites (P>0.05). The effects of age, gender and BMI on *WNT10A* methylation were investigated using the ANOVA GLM and we did not find any significant effects of these covariates (P>0.05).

Table 12: Difference between total MDS and methylation of specific CpG sites within WNT10A.

WNT10A **methylation**	**Lower MDS (0-4) Mean (SEM or SD) (n=20)**	**Higher MDS (5-14) Mean (SEM or SD) (n=20)**	**Unadjusted P-value[1]**	**Adjusted P-value[2]**
Total methylation	2.175 (0.348)	2.058 (0.347)	0.811	0.817
CpG site1[a]	4.905	4.405	0.4819	/
CpG site2[a]	0	0	>0.999	/
CpG site 3[a]	2.080	2.070	0.5075	/

[a] *Data is presented as medians, Mann-Whitney.*
[1] *T-test*
[2] *ANOVA GLM adjusted for age, gender, BMI, and smoking status*
Only for normally distributed data, the ANOVA GLM was calculated.

3.7 Differences in methylation of WNT pathway-related genes in participants at greater risk of CRC

The BFU Study participants included both healthy individuals and those who had adenomatous polyps removed at baseline in the BORICC Study, deemed at greater risk of CRC. For the purpose of primary analyses, participants were grouped together. As DNA methylation levels may vary in individuals at differing risk of CRC, the secondary analyses investigated whether participants with a prior history of adenomatous polyps had differential methylation levels of *WNT5A*, *WNT6* and *WNT10A*.

Methylation was quantified for all the three genes for both healthy participants and participants with polyps (Figure 13). Mean methylation for *WNT5A*, *WNT6*, and *WNT10A* across all participants was 8.04%, 1.82% and 2.66%, respectively. The highest mean methylation for participants with polyps was 6.82% (±1.79) for *WNT5A*, and the lowest was 1.16% (±1.14) for *WNT6*. For the healthy participants, again the mean methylation for *WNT5A* was the highest (8.39% ±2.03) and for *WNT6* the lowest (2.02% ±2.01). The difference in methylation between participants with and without adenomatous polyps was investigated for all the three genes using a t-test for normally-distributed data and the Mann-Whitney test for non-parametric data. Mean methylation of *WNT5A* was significantly lower in polyp (higher-risk) participants compared with the healthy group (P=0.042) (Figure 13).

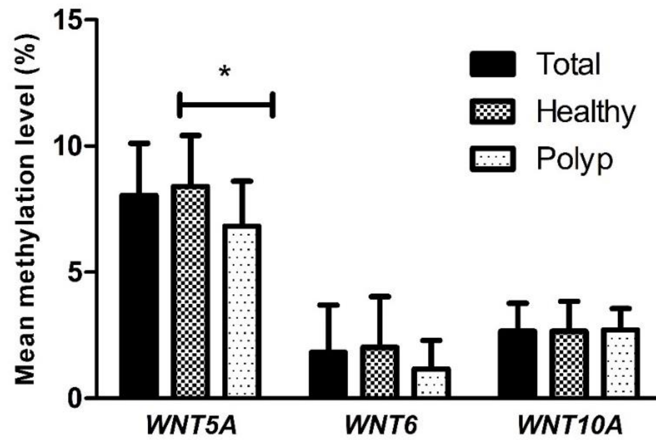

*Figure 13: Mean methylation (%) for WNT5A, WNT6, and WNT10A and mean methylation for healthy participants and participants with polyps for WNT5A, WNT6, and WNT10A. *P<0.05.*

3.7.1 Effects of adherence to the MD on methylation of WNT5A in healthy and polyp participants

Because *WNT5A* methylation differed significantly between healthy and polyp participants, we decided to further explore this difference to investigate whether this affected the response of healthy vs polyp participants to adherence to a MD. To do this, we re-ran the analyses described in sections 3.4.2 and 3.4.3 (investigating correlations between total MDS and *WNT5A* methylation and differences in methylation levels between lower and higher adherence groups) within the healthy and polyp participants separately. Within polyp participants, a trend for a strong positive correlation between MDS and mean *WNT5A* methylation was observed (Pearson's correlation coefficient= 0.594, P=0.092) (Figure 14).

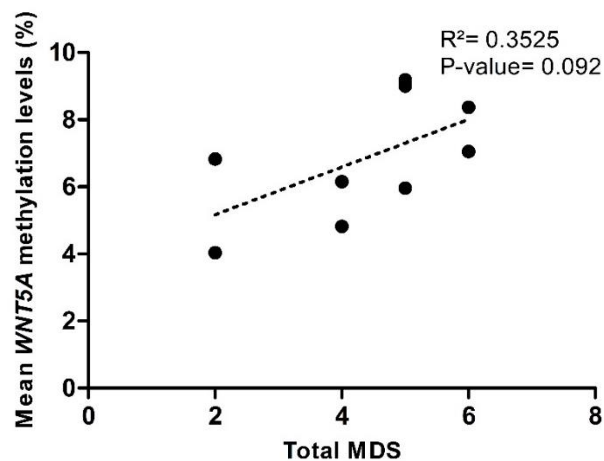

Figure 14: Correlation between MDS and mean WNT5A methylation across participants with polyps. Pearson correlation coefficient 0.594; P-value= 0.092

The differences in mean *WNT5A* methylation between lower and higher MDS groups was investigated for the healthy and polyp participants separately using t-tests. There were no significant differences between lower (mean 8.53%) and higher (mean 8.25%) MD adherers for mean *WNT5A* methylation within healthy participants (P>0.05). However, when investigating this in polyp participants, a significant difference in mean *WNT5A* methylation between lower (mean 5.46%) and higher (mean 7.91%) MD adherers was observed (P=0.032) (Figure 15). However, after adjusting for potential covariates such as age, gender, BMI, and smoking status this difference was no longer statistically significant. To investigate the interaction between the health status (healthy vs. polyp) and the MDS category for *WNT5A* methylation an ANOVA GLM was performed (data not shown). There was no significant interaction between health status and MDS category (P=0.129)

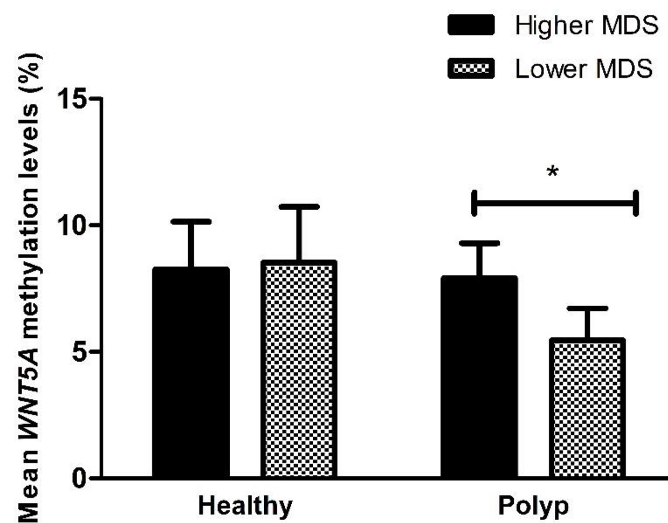

*Figure 15: Difference between total MDS and methylation of WNT5A for healthy participants and participants with polyps. * P<0.05*

4. Discussion

This project aimed to investigate the relationships between adherence to a MD and methylation of WNT pathway genes implicated in CRC. We hypothesized that the methylation of WNT pathway genes is modulated by adherence to the MD and differs between individuals with lower and higher MDS. We found no significant relationships between adherence to the MD and methylation at any of the CpG sites quantified or mean methylation of *WNT5A* or *WNT10A* genes. However, we observed a significant positive association between the MDS and the methylation at CpG site 5 quantified within *WNT6*. In addition, methylation across different CpG sites within each gene was mostly positively and significantly correlated and so using mean methylation across all CpG sites quantified is a good indicator of methylation for the whole gene.

To my knowledge, this is the first report of a positive correlation between methylation at CpG site 5 quantified within *WNT*6 and adherence to a MD pattern. In addition, a significant trend was observed for the differences in *WNT6* methylation at CpG site 5 between lower and higher MDS groups. Methylation at CpG site 5 appeared to be lower in participants with a lower MDS (mean methylation 0.59%) compared with those with a higher MDS (mean methylation 2.26%) (P=0.08). This evidence supports our hypothesis that methylation of *WNT6* for CpG5 is modulated by adherence to the MD and differs between individuals with lower and higher MDS. If methylation at this CpG site is correlated inversely with expression of the corresponding gene (as would be expected), this suggests that increased methylation and therefore reduced *WNT6* expression may occur with greater adherence to the MD.

In a study by Pampaloni *et al.* 2014, treatment of HCT-B8 cells with EVOO modulated the expression of a number of WNT family genes. HCT-B8 cells are colon cancer cell lines that have been engineered to overexpress estrogen receptor β. Treatment of HCT8-B8 cells with EVOO downregulated *WNT10A* and *WNT6* genes expression, while *WNT5A* was upregulated (53). There are several differences in the design of our study and that by Pampaloni *et al*. Firstly, this was an *in vitro* study in colorectal cancer cells whereas the current study was performed in macroscopically-normal rectal tissue from healthy participants. Secondly, Pampaloni and colleagues investigated the effects of one component of the MD, olive oil, compared with our study which assessed all components and assigned a MDS. Nevertheless, since higher EVOO intake is characteristic of MD adherence, the observed greater methylation with greater MDS is suggestive of reduced *WNT6* expression, which is in agreement with that observed by Pampaloni et al (53). These findings must be interpreted with caution as most studies report

mean methylation levels across all quantified CpG sites, whereas our findings are for CpG site 5 within a specific primer sequence (PM000979720). Because in this study commercially available CpG assays were used, we cannot say with any certainty that our CpG sites lay within the promotor region and therefore influence the expression of the related gene.

As DNA methylation levels may vary in individuals at differing risk of CRC, *post hoc* analyses investigated whether participants with a prior history of adenomatous polyps had differential methylation levels of *WNT5A*, *WNT6* and *WNT10A* and may therefore respond to a different extent to the MDS. We did not find differences in *WNT6* or *WNT10A* methylation levels between healthy (normal-risk) and polyp (higher risk) participants. A higher level of *WNT10A* hypermethylation has previously been reported in CRC samples than in controls (54). Farkas *et al.* 2014 analysed methylation status in 12 paired colorectal tumors and adjacent healthy mucosal tissues. The results showed a significant differential methylation in 11 genes of the Wnt/β-catenin pathway, whereof one of these genes was *WNT6* (55). Our results showed that mean *WNT5A* methylation was significantly lower in polyp (higher-risk) participants compared with the healthy group, suggesting increased *WNT5A* expression. The role of WNT5A in colorectal tumorigenesis is not fully understood. Previously, Ying *et al.* examined the expression and methylation of *WNT5A* in normal colonic tissue, CRC cell lines and tumors. *WNT5A* is silenced in most CRC cell lines due to promoter hypermethylation but is expressed in most normal tissues including the colon and is unmethylated in normal colon epithelial cells. *WNT5A* is frequently inactivated in CRC by tumor-specific methylation (56). Another study showed that there was an association between *WNT5A* methylation and increasing tumor stage (57).

Further post hoc analyses revealed a trend for a strong positive correlation between MDS and mean *WNT5A* methylation within polyp (higher risk) participants only. No such relationship was observed when investigating this only in healthy participants. For participants with polyps, methylation of *WNT5A* was almost 50% lower in those with lower MDS compared with participants with higher MDS. However, after adjusting for potential covariates such as age, gender, BMI, and smoking status this difference was no longer statistically significant. To our knowledge, this study is the first to report findings on relationships between adherence to the MD and *WNT5A* methylation. *WNT5A* is involved in activating several noncanonical Wnt signaling pathways, which can inhibit or activate canonical Wnt/β-catenin signalling pathway in a receptor context-dependent manner. Aberrant activation or inhibition of *WNT5A* signalling

is emerging as an important event in cancer progression, exerting both oncogenic and tumor suppressive effects (24, 58). Ying *et al.* 2008 reported that *WNT5A* is frequently epigenetically inactivated in CRC and additionally, they stated that *WNT5A* expression resulted in significant suppression of colony formation of CRC cells, that is at least partially mediated by Wnt/β-catenin signalling pathway (56).

4.1 Study strengths and limitations

This study has several strengths. First, to our knowledge, this was the first study that investigated the relationship between adherence to a MD and methylation of WNT pathway genes (*WNT5A*, *WNT6*, and *WNT10A*) implicated in CRC. Second, the obtaining of the MDS for each participant was adapted from Martínez-González *et al*. 2015**,** considered a reputable and effective method which has been utilised in a number of studies prior to this one. Therefore, it can be assured that it provides an accurate measure of adherence to the MD pattern. Third, the BFU Study participants were extensively phenotyped and were largely representative of the population in the North of England in terms of diet and other lifestyle factors. Another strength of this study is that the methylation measurements were made in the target tissue itself (the colorectal mucosa).

One of the limitations of this study is the self-report method of the dietary assessment whereby participants can over- or underestimate their consumption of particular dietary components, and this can result in a misclassification and may explain the lack of significant associations. Another limitation of this study is that the MDS data were dichotomised at the median to create lower and higher MDS groups. This was done because of the small range of MDS of the participants in this study. Therefore, because overall the BFU Study participants had a lower MDS, the lower and higher groups that were used in this study may not equate to lower and higher risk of CRC. Additionally, another limitation of this study is the relatively small sample size (n=40) which could result in insufficient statistical power to determine any potential relationships between adherence to the MD and methylation of genes involved in the WNT pathway and which increases the risk of a type II error.

4.2 Future work

The next step in this research is to investigate the changes at mRNA level for each target gene to investigate whether DNA methylation regulates gene expression, and whether this is associated with adherence to the MD. Further work would aim to address some of the

aforementioned limitations. Future larger studies are required to investigate these relationships in a UK population with sufficient statistical power. In future research, it would be interesting to investigate WNT pathway gene methylation from participants with a broader range of MDS**.** As well, in future research different WNT pathway genes could be included then the ones that are investigated in this study.

5. Conclusion

To our knowledge, this is the first study to investigate the relationships between adherence to a MD and methylation of WNT pathway genes implicated in CRC; *WNT5A, WNT6,* and *WNT10A.* We found no evidence to support the hypothesis that MD adherence is associated with *WNT10A* or *WNT5A* methylation. However, methylation of *WNT6* at CpG5 correlated with MD.

A post hoc analysis showed evidence of a significant difference in methylation levels of *WNT5A* between healthy and participants with a history of adenomatous polyps. In addition, evidence showed that *WNT5A* methylation was influenced by adherence to the MD in participants with polyps. For participants with polyps, methylation of *WNT5A* was almost 50% lower in those with lower MDS compared to those with higher MDS suggesting that the MD modulates the methylation in participants with a higher CRC risk.

Despite the few limitations of this study, it has played an important role in identifying and filling a gap in the literature, as studies investigating the molecular mechanisms underlying the effects of diet and lifestyle, such as MD patterns, on CRC risk are limited. Further research is needed to elucidate the relationship between WNT pathway genes and adherence to a MD, especially in those at higher CRC risk.

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<u>Supplemental information</u>

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Supplement 1: DNA extractions

The mean concentration for the rectal DNA concentrations is respectively 187.57 ng/µl with an IQR of 99.3 ng/µl. The lowest and highest concentration measured are respectively, 50.2 and 693.5 ng/µl. The DNA concentrations of all the rectal biopsies are listed in table in the supplement.

Supplement 2: DNA concentrations

Table 13: DNA concentration from rectal mucosal biopsies.

ID	Concentration (nq/µl)	A260/280	A260/230
051	693.5	1.89	2.25
050	140.4	1.89	2.4
028	107.3	1.75	1.44
003	532.2	1.6	1.6
123	70.6	1.7	1.25
199	127.4	1.78	1.58
022	97.5	1.81	1.85
151	50.2	1.81	1.78
200	150	1.75	1.49
262	83.3	1.86	2.33
263	129.1	1.88	2.32
226	183	1.87	2.27
175	113.9	1.87	2.27
145	55.2	1.89	2.32
142	219.4	1.88	2.21
154	306.3	1.89	2.23
141	96.8	1.85	1.89
P002	154.3	1.87	2.1
239	340.7	1.89	2.23
P001	88.1	1.72	1.33
247	187.8	1.87	2.05
208	244.4	1.87	2.2
P012	106.7	1.85	2.12
074	190.3	1.84	1.97
P077	198.8	1.88	2.17
023	169.7	1.86	2.13
P085	197.4	1.81	1.73
015	159.8	1.84	2.11
241	192.6	1.87	2.03
250	467.4	1.88	2.27
P088	99.9	1.78	1.52
P025	79.2	1.79	1.65
P039	380.3	1.88	2.17
124	64.1	1.83	1.8
096	140	1.86	2.06
257	231.1	1.84	1.83
234	280.2	1.85	2.09
045	107.9	1.84	2.05
P043	106.2	1.84	2.09
008	159.7	1.87	2.14

Supplement 3: Thermal cycler steps for bisulphite modification

Table 14: Thermal cycler steps

Step	Temperature (°C)	Duration
1	98	10 minutes
2	64	2.5 hours
3	4	Storage up to 20 hours

Supplement 4: Testing annealing temperatures

A PCR test run with a temperature gradient from 48 °C to 60 °C was performed for each primer to test the annealing temperature. For all the primers an annealing temperature of 50.4 °C was chosen. (Figure 16, Figure 17, and Figure 18)

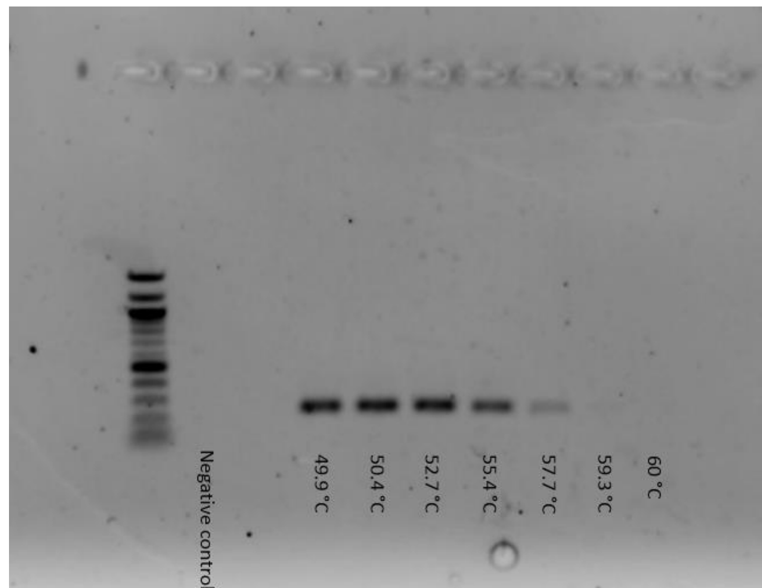

Figure 16: Image of gel electrophoresis to test annealing temperature for WNT6.

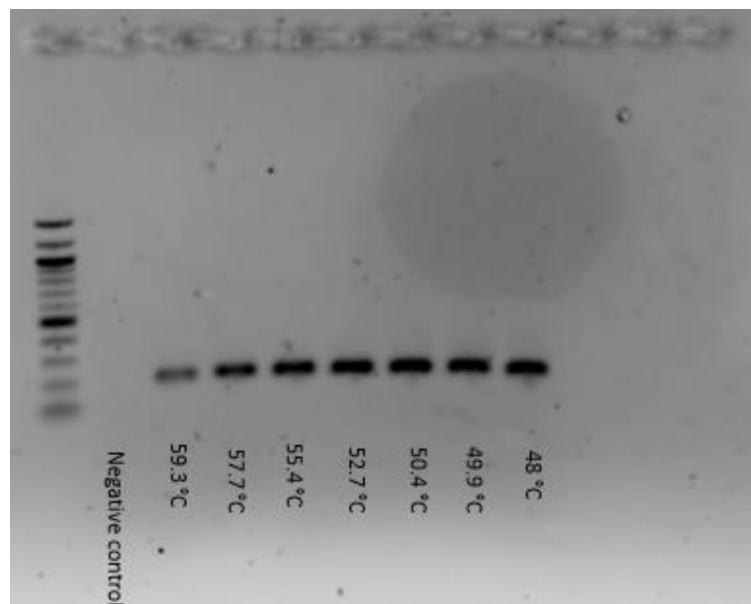

Figure 17: Image of gel electrophoresis to test annealing temperature for WNT5A.

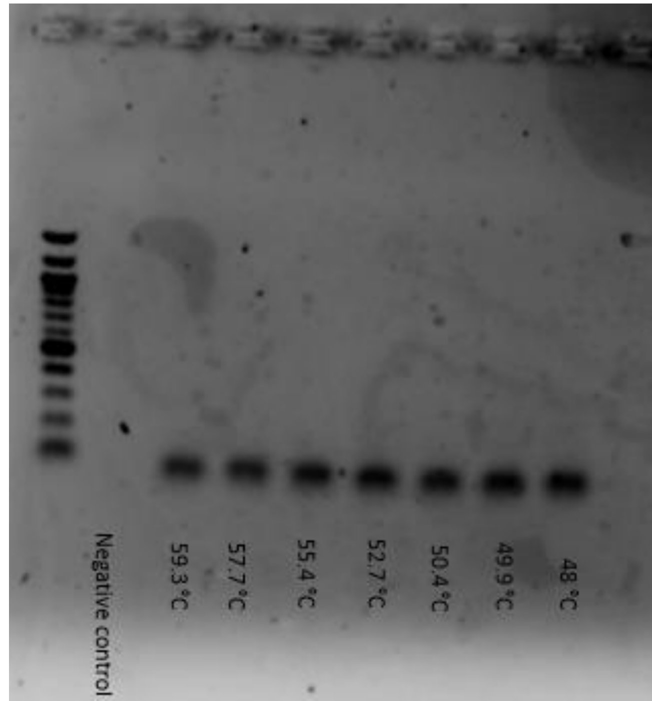

Figure 18: Image of gel electrophoresis to test annealing temperature for WNT10A.

Supplement 5: Quality assurance of PCR products by gel electrophoresis

Agarose gel (1%) electrophoresis was performed to verify if each gene of interest had been successfully amplified in the absence of contamination (Figure 19, Figure 20, and Figure 21) SYBR™ Safe DNA Gel stain was used to stain the gel for visualisation of the bands. DNA ladder (New England Biolabs, USA) was pipetted into the first well for reference. 2µl of PCR product was pipetted into each of the remaining wells. The gel was run at 100V for 30 minutes with a current of 400. The images of the gels were captured using the LI-COR® (LI-CORE Biosciences, USA) software.

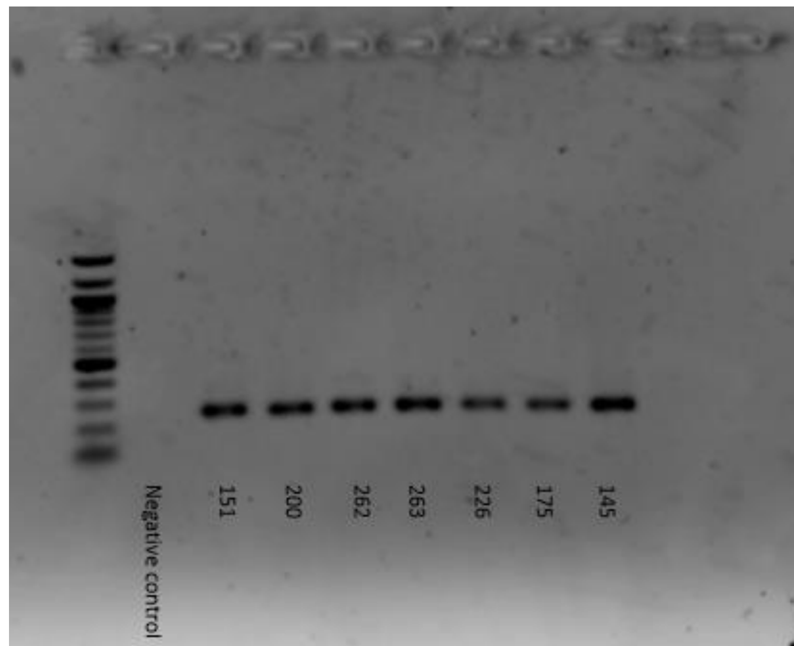

Figure 19: Image of gel electrophoresis for WNT6.

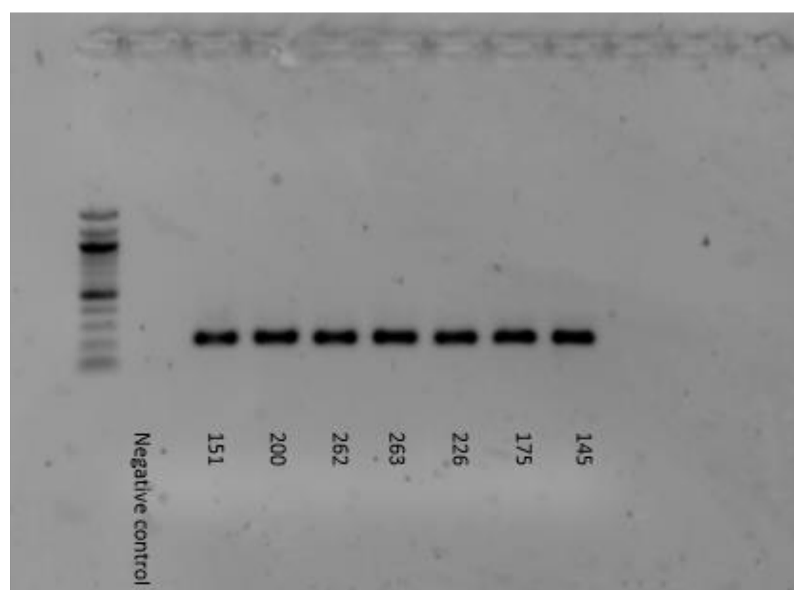

Figure 20: Image of gel electrophoresis for WNT5A.

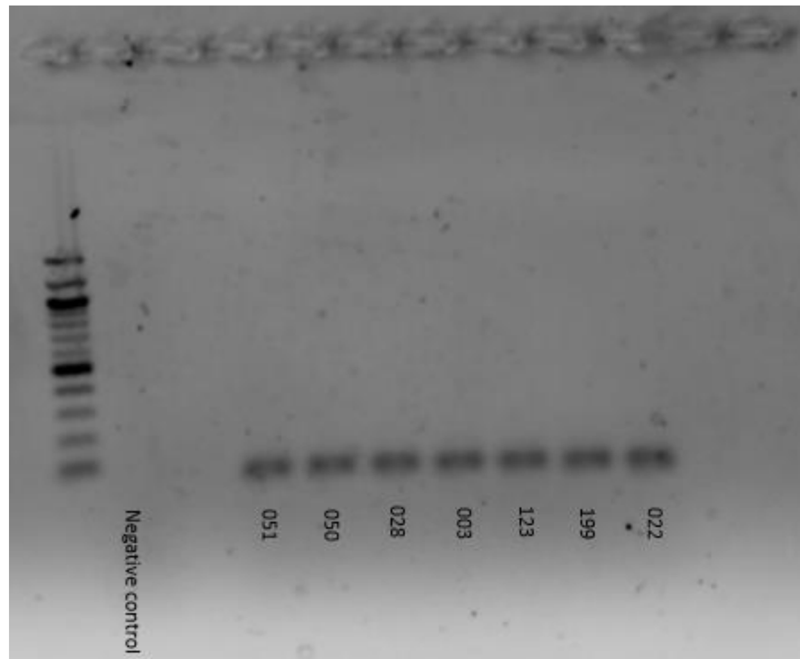

Figure 21: Image of gel electrophoresis for WNT10A.

Supplement 6: Correlations between Mediterranean diet and methylation across all CpG sites of all genes

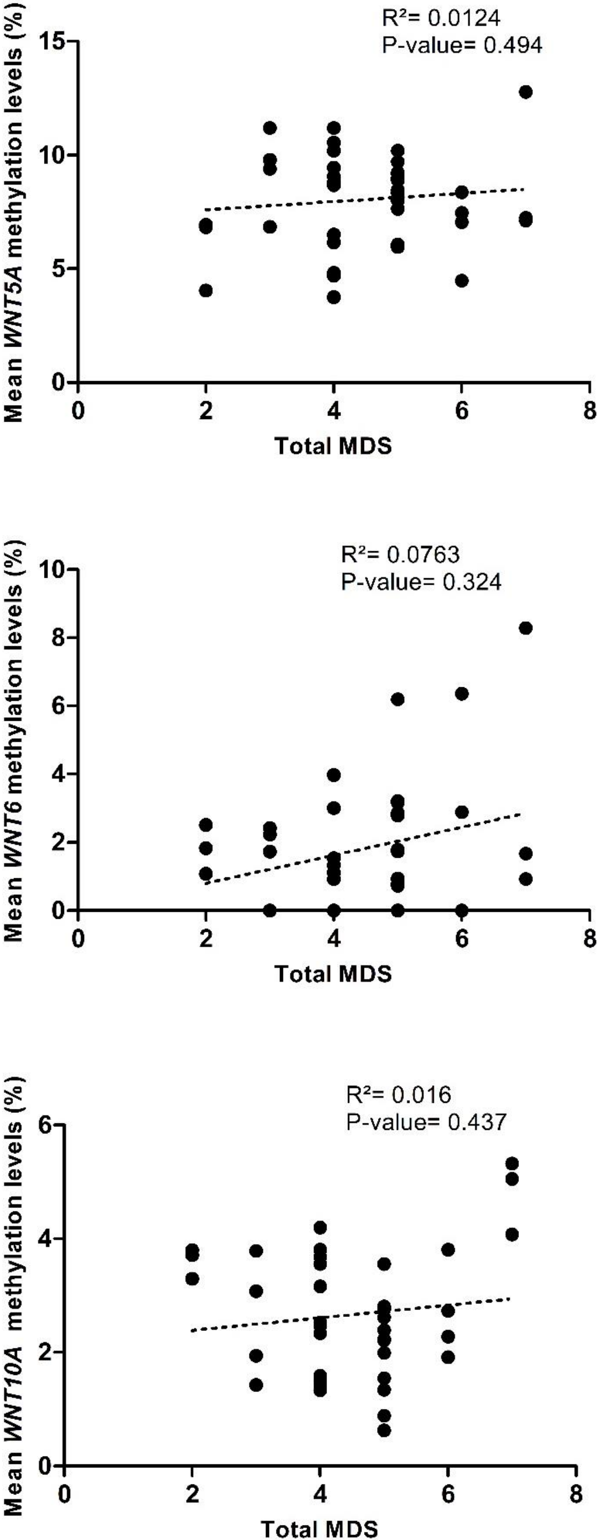

Figure 22: Correlation between MD score and mean methylation across all CpG sites for WNT5A, WNT6, and WNT10A. The upper figure displays WNT5A. The Pearson correlation coefficient was 0.111 and the P-value was 0.494. The middle figure displays WNT6. The Spearman Rho correlation coefficient was 0.378 and the P-value was 0.016. The lower figure displays WNT10A. The Pearson correlation coefficient was 0.126 and the P-value was 0.437.