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# Vitamin D intake is negatively associated with promoter methylation of the Wnt antagonist gene *DKK1* in a large group of colorectal cancer patients

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## Abstract

Diet and lifestyle help mediate colorectal cancer (CRC) risk but the molecular events that mediate these effects are poorly characterized. Several dietary and lifestyle factors can modulate DNA methylation suggesting that they may influence CRC risk through epigenetic regulation of cancer-related genes. The Wnt regulatory genes *DKK1* and *Wnt5a* are important contributors to colonic carcinogenesis and are often silenced by promoter hypermethylation in CRC; however, the dietary contributions to these events have not been explored. To investigate the link between dietary/lifestyle factors and epigenetic regulation of these Wnt signaling genes, we assessed promoter methylation of these genes in a large cohort of Canadian CRC patients from Ontario (n=549) and Newfoundland (n=443) and examined associations to dietary/lifestyle factors implicated in CRC risk and/or DNA methylation including intake of vitamins, fats, cholesterol, fibre, and alcohol as well as BMI and smoking status. Several factors were associated with methylation status including alcohol intake, BMI, and cigarette smoking. Most significantly however, dietary vitamin D intake was strongly negatively associated with *DKK1* methylation in Newfoundland (p=0.001) and a similar trend was observed in Ontario. These results suggest that vitamin D and other dietary/lifestyle factors may alter CRC risk by mediating extracellular Wnt inhibition.

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## 1. Introduction

Over the past several decades, epidemiological studies have underscored the importance of dietary and lifestyle factors on colorectal cancer (CRC) risk. Among the supported environmental risk factors for CRC are obesity, smoking, consumption of red meats and fats, and low intake of fruits and vegetables. Despite potential roles for these factors in CRC onset, little is known about the specific molecular events that may mediate these changes in tumor biology.

A broad mechanism with emerging importance by which these and other factors induce their risk-modifying effects is via DNA methylation. Methylation of CpG dinucleotides within gene promoters is a fundamental method of transcriptional silencing in eukaryotes and altered genomic methylation status is common to many cancers. In fact, aberrant promoter hypermethylation is a particularly well-established hallmark of CRC, defining the CpG island methylator phenotype (CIMP) and driving onset of most microsatellite unstable tumours through silencing of the critical DNA mistmatch repair gene *MLH1*. Several dietary and lifestyle risk factors for CRC are known to influence DNA methylation levels within the colon including folate, alcohol, and certain B vitamins [1]; however, the specific risk genes affected remain poorly characterized.

Among the most important genes in colorectal cancer are those encoding mediators of Wnt signaling. Canonical Wnt signaling mediates homeostatic proliferation in colonic crypts and hyperactivation of this pathway is the major driver of colorectal tumour growth. Diet-induced changes in Wnt activity within the colon have been observed during both development [2] and tumourigenesis [3] but the involvement of promoter methylation within Wnt genes in causing these important changes in Wnt activity has not been explored.

The *DKK1* and *Wnt5a* genes encode pivotal Wnt mediators that are silenced in early CRC by promoter hypermethylation [4, 5]. *DKK1* encodes a canonical Wnt suppressor that has strong anti-proliferative and pro-apoptotic effects in CRC cell lines [6, 7] and dramatically reduces tumour burden in mouse xenografts [7]. *Wnt5a* encodes a quintessential non-canonical Wnt ligand that has emerging roles in both colonic tumour-suppression and oncogenesis (reviewed in [8]). Expression levels of *DKK1* and *Wnt5a* have been associated with disease outcome in several cancers including CRC [9-11] bringing into question whether epigenetic silencing of these genes may be responsible for changes in CRC risk. To investigate the potential role of *DKK1* and *Wnt5a* promoter methylation in mediating diet-associated changes in CRC risk, we quantified promoter methylation levels of these genes in two large populations of CRC patients from Canada and examined their associations to dietary and lifestyle factors implicated in CRC risk and/or colonic methylation.

## 2. Materials and Methods

### 2.1 Study Participants

Participants included in this study were accrued through the population-based Ontario Familial Colorectal Cancer Registry (OFCCR) and Newfoundland Familial Colorectal Cancer Registry (NFCCR). Patient accrual, data collection, and biospecimen collection

procedures at OFCCR have been previously detailed [12]. Briefly, Ontario residents diagnosed with pathology-confirmed CRC between the ages of 20 and 74 from 1997 to 2000 were eligible for recruitment. Apparent cases of familial adenomatous polyposis were excluded and recruitment was preferential for cases with positive familial risk based on patient response to a family history questionnaire. 1004 probands satisfying these conditions had blood and/or tissue biospecimens available at the time of study accrual. Due to the high prevalence of Caucasians (92.5%) and to limit the possible effects of population stratification, non-white patients and those with unknown or mixed ethnic background were excluded from this study. Of these 921 (91.7%) probands, 561 (55.8%) had tumour samples available for methylation analysis. Several probands were excluded during methylation analysis due to poor quality DNA (see Methods: *MethyLight*), leading to 545 (54.2%) final cases. An overview of patient clinicopathological features is provided in Table 1.

Patient accrual at NFCCR was similar to that of OFCCR and has also been described in detail [13]. Briefly, cases were accrued over a slightly later recruitment period (1999 to 2003) and included all incident CRC patients irrespective of family history. In addition, for deceased patients, NFCCR accepted proxy consent from living family members, which led to more frequent inclusion of late-stage patients. Of the 747 probands recruited by NFCCR at the time of study accrual, 721 (96.5%) probands were Caucasian (northern European origin) and had tumour samples available for methylation analysis. Methylation analysis was successfully performed on 687 (92.0%) cases after removal of poor quality samples. 253 (35.8%) probands were recruited through proxy consent of family members and removed from the analysis to increase the accuracy of dietary reporting.

### 2.2 Accrual, Selection, and Definition of Dietary and Lifestyle Variables

Dietary and lifestyle data for each population was abstracted through patient responses to two mailed questionnaires: personal history (PHQ) and food frequency (FFQ). The FFQ in Ontario (ON) was developed by the Epidemiology Program at the Cancer Research Center of Hawaii and has been previously described and validated [14]. The FFQ in Newfoundland (NFLD) was developed at Memorial University, St. John's, Newfoundland [15]. Several probands from each population did not respond to the FFQ (ON=35, NFLD=59) or PHQ (ON=39, NFLD=5). Dietary and lifestyle variables were selected for abstraction based on a literature search of risk factors for CRC and/or colonic methylation. Dietary variables examined were: vitamins B2, B6, B12, and D, folate, isoflavonoids, fibre, fat, saturated fat, and cholesterol. Lifestyle variables examined were: daily intake of alcohol (averaged over drinkable lifetime), history of cigarette smoking, and body mass index (BMI). Dietary fat and saturated intakes were reported in absolute (daily intake in weight units) and/or relative (daily intake as percent of total daily calories) format.

Dietary and lifestyle habits in Ontario and Newfoundland are considerably discordant. To remove possible bias due to outliers and to improve homogeneity between populations, all nutrient intake measurements were categorized into population-specific rounded quartiles except for vitamin D intake from supplements, which was categorized into users and non-users due to a limited number of users. Daily intake of alcohol (previously defined [16]) was grouped by median intake after segregation of never-drinkers, who were assigned an intake

value of zero. Smoking status (previously defined [16]) was grouped into ever- and never-smokers. BMI was examined at age 20 ("BMI Age 20") and at one year prior to cancer diagnosis ("BMI current") and categorized according to World Health Organization guidelines with collapsed groups where appropriate to ensure statistical power. For sex-stratified and stage-stratified analyses of vitamin D intake, the upper three quartiles of intake were collapsed into a single comparison group to ensure statistical power.

### 2.3 Methylation Analysis of Candidate Genes

*DKK1* and *Wnt5a* were selected as candidate genes based on their strong functional effects on Wnt signaling and previous associations with prognostically-relevant CRC subtypes. Methylation analysis of two other Wnt genes, *SFRP1* and *WIF-1*, was also performed; however, these genes were not considered for analysis due to a limited number of unmethylated individuals in Ontario (*SFRP1*=28; *WIF-1*= 64) and Newfoundland (*SFRP1*=21; *WIF-1*= 39).

Methylation analysis was performed on DNA extracted from paraffin-embedded tumours using semi-quantitative MethyLight assay, as previously described [17]. Primers and probes were designed to amplify the promoter CpG island regions of *DKK1* and *Wnt5a* [7, 18]. The extent of promoter methylation was calculated as percent methylated reference (PMR) as follows: PMR $=[\text{gene/reference}]_{\text{sample}} / [\text{gene/reference}]_{\text{CpGenome}} \times 100\%$ [19]; where CpGenome DNA is a fully methylated positive control. *Alu-C4* was used as the internal reference control amplicon, as recommended to maximize assay robustness [20]. For cases with multiple DNA samples due to synchronous baseline tumours (ON: n=34, NFLD: n=16), PMR values were averaged. Cases were categorized as "methylated" or "unmethylated" based on a tumour-specific methylation cutoff of PMR ≥ 10% as previously validated [5, 21]. Samples with PMR values ±2% of this cutoff value were reanalyzed and PMR values were averaged to more accurately define methylation status.

Validation was also performed on randomly selected samples to assess batch effects. PMR readings fluctuated <10% among different batches. During both screening and validation, samples in which the *Alu-C4* internal control reaction reached threshold fluorescence above 22 cycles (C(t) > 22) were considered to be poor quality, retreated with sodium bisulphite, and subsequently reanalyzed to ensure robust amplification. Samples that remained poor quality in both independent analyses were removed from the analysis (ON: n=16; NFLD: n=34).

### 2.4 Statistical Analysis

Associations of dietary and lifestyle exposure variables to methylation status of *DKK1* or *Wnt5a* (outcome variables categorized as "methylated" versus "unmethylated", as detailed above) were assessed by unconditional logistic regression. Logistic regression models were adjusted for age, sex, and total energy intake. Adjustment for other clinical and pathological features was not performed in order to avoid overadjustment for factors not expected to impact the direct biological relationship between diet and methylation status. Two-sided $p < 0.05$ was considered statistically significant. Exposure variables were carefully selected from evidence of effects on colorectal cancer risk and/or gene methylation status; as a result,

statistical adjustment for multiple hypothesis testing was not performed. All analyses were performed using SPSS v16.0 (SPSS Inc.).

## 3. Results

We examined the associations of dietary factors (Table 2) and lifestyle factors (Table 3) with methylation status of *DKK1* and *Wnt5a* in 549 CRCs from Ontario and 443 CRCs from Newfoundland. There were no significant differences in the mean age at diagnosis, tumor stage, location, histological type, or methylation status of *DKK1* or *Wnt5a* between these populations. However, in Newfoundland compared to Ontario there were a higher proportion of males (p=0.005), lower proportion of MSI tumor ($p<10^{-}6$), and lower proportion of high grade tumors (p=0.002).

Several dietary and lifestyle factors were significantly associated with promoter methylation status of *DKK1* or *Wnt5a*. Most significantly, among Newfoundland CRCs there was a strong negative association *DKK1* methylation and overall vitamin D intake (p=0.001). Upon further stratification by intake source, a similar negative relationship was found between *DKK1* methylation and vitamin D intake from dietary sources (p=0.001) and from vitamin supplements, although the latter did not reach statistical significance (p=0.11). *Wnt5a* methylation was also negatively associated with overall vitamin D intake in this population (p=0.05) and the directionality of this relationship was maintained when examining vitamin D intake from dietary sources (p=0.11). To further investigate this borderline relationship, individuals with low vitamin D intake (first quartile) were compared to individuals with high vitamin D intake (other quartiles). Individuals with high intake were significantly less likely to have *Wnt5a* methylation (p=0.04, OR=0.49 [0.25, 0.96]). In Ontario, vitamin D intake was not significantly associated with methylation of either gene, although directionality of this relationship was similar when examining overall/dietary vitamin D intake and *DKK1* methylation as well as supplemental vitamin D intake and *DKK1* methylation but not dietary vitamin D intake.

To investigate whether gender differences between Newfoundland and Ontario may explain the discordant relationships between vitamin D intake and *DKK1* methylation in these populations, a sex-stratified analysis was conducted. In Newfoundland, the inverse association between overall vitamin D intake and *DKK1* methylation were both maintained for males alone (p=0.03) and for females alone (p=0.001) although this relationship seemed stronger in females (Supplementary Table 1). Similar results were seen for dietary vitamin D intake and *DKK1* methylation. In Ontario, vitamin D intake remained unassociated with *DKK1* methylation in both sexes. Another possible reason behind the discordant vitamin D findings could be differential accumulation of stage-specific alterations that change the ability of vitamin D to influence *DKK1* methylation. To examine this hypothesis, individuals in each population were stratified into early tumor stage (I/II) and late tumor stage (III/IV) and an analysis was conducted. In Newfoundland, overall vitamin D intake remained negatively associated with *DKK1* methylation in early stage (p=0.003) and late stage tumors (p=0.006) (Supplementary Table 2). In Ontario, overall vitamin D intake became negatively associated with *DKK1* methylation in early stage tumors (p=0.05) but not in late stage tumors (p=0.87).

Other factors that showed a modest association with methylation status of *DKK1* or *Wnt5a* in Ontario include: negative associations between *DKK1* methylation and current BMI (p=0.05), *Wnt5a* methylation and cigarette smoking (p=0.04), and *Wnt5a* methylation and daily alcohol intake (p=0.02). Positive associations were found between *Wnt5a* methylation and BMI at age 20 (p=0.02) as well as *Wnt5a* methylation and percent of daily calories from saturated fat (p=0.03). None of these comparisons reached statistical significance in Newfoundland; however, the negative directionality between *Wnt5a* methylation and smoking was consistent. No associations were found between methylation status and intake of B vitamins, cholesterol, fibre, folate, isoflavonoids, or other measurements of fat in either population.

## 4. Discussion

In order to fully understand the roles of diet and lifestyle in cancer risk and progression and to improve related guidelines for cancer prevention and treatment, there is a need to identify specific, functionally-relevant molecular events that may be responsible for these outcomes. Vitamin D is an emerging protective factor for CRC [22] that mediates expression of numerous cancer genes through conventional binding of the internalized vitamin D receptor to gene promoters or through epigenetic mediation of these gene promoters including histone modification and gene methylation [23]. We found that vitamin D intake, both overall and from dietary sources, was a strong negative indicator of *DKK1* promoter methylation in Newfoundland CRCs. Due to the silencing effect of promoter methylation on *DKK1* gene expression [6], this negative association suggests that *DKK1* is preferentially expressed in CRC patients with high vitamin D intake. Vitamin D has previously been shown to induce *DKK1* expression in CRC cells and in corresponding mouse xenografts through a mechanism independent of canonical promoter recruitment [24]. Consequently, our results suggest that the mechanism involved may be loss of *DKK1* promoter methylation and that this mechanism may also be found in primary CRC. Due to the tumour suppressive functions of DKK1, this epigenetic event may represent an important molecular link between vitamin D and CRC protection. Interestingly however, *DKK1* methylation was not significantly associated with vitamin D intake derived from vitamin supplements. The significance of this result is unknown due to the methods of data ascertainment used in this study but should be further explored to determine whether certain types of vitamin D may selectively influence methylation at the *DKK1* promoter.

Vitamin D and *DKK1* methylation were not significantly associated in Ontario CRCs despite this relationship maintaining similar directionality in both populations. Ontario and Newfoundland are clinically and genetically heterogeneous CRC populations that vary with respect to disease incidence and mortality [Health Canada, 25], family history [13], and tumour MSI status [26]. It is likely that components of the genetic and environmental variability that underlie these population-specific differences may also contribute to the discordant relationship between vitamin D and *DKK1* methylation found in this study. Further population stratification suggested that this discordance is not due to gender differences but may be related to tumor stage in Ontario since early stage tumors but not late stage tumors exhibited the negative association between *DKK1* methylation and overall vitamin D intake. Perhaps a specific alteration occurs during tumor development in Ontario

that makes these individuals selectively fail to respond to vitamin D intake. Further genetic analysis of vitamin D metabolizing enzymes in this population may help to address this observation.

Differences in sunlight exposure may also influence the effect of measured vitamin D intake on methylation due to the vital role of UV-B radiation in catalyzing endogenous synthesis of the majority of this nutrient in individuals with adequate sunlight exposure. Since UV exposure in Ontario is abundant and much higher than in Newfoundland [27], vitamin D intake from dietary sources may be of limited biological significance among Ontario CRCs. This may help explain the limited significance of the relationship between measured vitamin D intake and *DKK1* methylation in Ontario. Analysis of circulating 1,25-dihydroxyvitamin D levels may help to more directly scrutinize the relationship between vitamin D and *DKK1* methylation in Ontario CRCs.

Other dietary and lifestyle factors for CRC that were weakly associated with methylation status of *DKK1* or *Wnt5a* in Ontario included cigarette smoking and alcohol as negative indicators while BMI showed opposing associations with methylation status. The significance of these results varied by gene and method of data representation (e.g. alcohol intake measured in grams versus percent of daily calories). Nevertheless, the negative relationship between smoking and methylation is consistent with smoking as a negative indicator of Wnt antagonist promoter methylation in lung cancer [28] but inconsistent with the general methylation-promoting effects of smoking including its strong association with CIMP CRCs [29] – where the majority of *DKK1* and *Wnt5a* methylation is found [4, 5]. Furthermore, it is unclear the extent to which this negative relationship between smoking and methylation may reflect increased expression of these genes since tobacco seems to have alternate mechanisms of silencing Wnt antagonist genes [30]. Additional functional analyses examining the effects of smoking on methylation and expression of Wnt antagonist genes will be required to assess the possible contribution of these epigenetic events to smoking-related changes in CRC risk.

Like smoking, BMI has been associated with CIMP CRC; however, BMI and several other measures of body mass have also been linked to risk of non-CIMP CRCs [31]. The relationship between body mass and methylation within CRC is therefore less certain than that of smoking. Conversely, alcohol has been linked to hypomethylated CRC via *LINE-1* status [32] but is not correlated with CIMP status [33]. Consequently, it is uncertain whether the observed associations of BMI and alcohol with Wnt antagonist methylation may be related to their broader relationships to overall CRC methylation. Further scrutiny of these factors as specific mediators of Wnt antagonist gene methylation and expression will be necessary to determine whether these genes play a significant role in BMI-related or alcohol-related changes in CRC risk.

This is the first study to investigate the dietary and lifestyle contributions to promoter methylation of important Wnt regulatory genes in CRC, offering insight into the functionally-relevant epigenetic events through which these factors may influence CRC risk and outcome. We found a strong negative association between vitamin D intake and *DKK1* methylation, providing a possible epigenetic link between vitamin D and CRC protection.

Smoking, alcohol intake, and BMI were also modestly linked to methylation status; however, due to the lack of consistent findings between our populations, additional validation in other CRC cohorts is warranted. Nevertheless, this study paves the way for further examination of Wnt antagonist gene methylation in order to assess the contribution of these events to dietary and lifestyle-associated changes in CRC risk.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **CRC** | colorectal cancer |
| **CIMP** | CpG island methylator phenotype |
| ***DKK1*** | dickkopft 1 homolog |
| ***MLH1*** | mutL homolog 1 |
| **MSI** | microsatellite instability |
| **NFCCR** | Newfoundland Familial Colorectal Cancer |
| **OFCCR** | Ontario Familial Colorectal Cancer Registry |
| **PMR** | percent methylated reference |
| ***Wnt5a*** | wingless-related MMTV integration site 5A |

## Table 1

Overview of clinicopathological features among colorectal cancer cases from Ontario and Newfoundland.

| | No. of Cases (%) | |
|---|---|---|
| | **Ontario** | **Newfoundland** |
| **Cases of primary colorectal carcinoma** | 549 | 443 |
| **Mean Age (± Std.Dev.)** | 60.6 ± 8.8 | 61.3 ± 9.4 |
| **Sex** | | |
| Female | 263 (47.9) | 172 (38.8) |
| Male | 286 (52.1) | 271 (61.2) |
| **Tumor Location**[a] | | |
| Proximal | 209 (38.1) | 175 (39.5) |
| Distal | 332 (60.5) | 260 (58.7) |
| Unavailable/Overlapping | 8 (1.5) | 8 (1.8) |
| **TNM Stage** | | |
| 1 | 96 (17.5) | 88 (19.9) |
| 2 | 211 (38.4) | 177 (40.0) |
| 3 | 168 (30.6) | 141 (31.8) |
| 4 | 30 (5.5) | 37 (8.4) |
| Unavailable | 44 (8.0) | 0 (0.0) |
| **Histological Grade** | | |
| Low | 43 (7.8) | 66 (15.1) |
| Moderate | 392 (71.4) | 331 (74.7) |
| High | 64 (11.7) | 38 (8.6) |
| Unavailable | 50 (9.1) | 7 (1.6) |
| **Histological Type**[b] | | |
| Non-mucinous | 446 (81.2) | 395 (89.2) |
| Mucinous | 64 (11.7) | 48 (10.8) |
| Unavailable | 39 (7.1) | 0 (0.0) |
| **MSI Status** | | |
| Stable | 385 (70.1) | 368 (83.1) |
| Low | 71 (12.9) | 20 (4.5) |
| High | 88 (16.0) | 52 (11.7) |
| Unavailable | 5 (0.9) | 3 (0.7) |
| ***DKK1* Methylation** | | |
| Unmethylated | 477 (87.0) | 382 (86.2) |
| Methylated | 71 (13.0) | 61 (13.8) |
| ***Wnt5a* Methylation** | | |
| Unmethylated | 438 (79.8) | 361 (81.5) |
| Methylated | 107 (19.5) | 75 (16.9) |
| Unavailable | 4 (0.7) | 7 (1.6) |

[a] Locations proximal or distal to the splenic flexure. Tumors at the splenic flexure are included in the “Proximal” category.

[b] Presence of any mucin within the tumor stroma is defined as “Mucinous”.

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## Table 2

Associations between dietary factors and methylation status of *DKK1* and *Wnt5a* in colorectal carcinomas from Ontario and Newfoundland. Models were adjusted for age, sex, and total energy intake.

| | | Ontario | | | | | | Newfoundland | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *DKK1* | | *Wnt5a* | | | | *DKK1* | | *Wnt5a* | |
| **Variable** | **Total (%)** | **OR (95% CI)** | **p** | **OR (95% CI)** | **p** | **Variable** | **Total (%)** | **OR (95% CI)** | **p** | **OR (95% CI)** | **p** |
| **Vitamin D (ug)** | | | | | | **Vitamin D (ug)** | | | | | |
| Overall | | | 0.25 | | 0.76 | Overall | | | 0.001 | | 0.05 |
| 0-4 | 132 (25.7) | 1.00 (Referent) | | 1.00 (Referent) | | 0-4 | 101 (26.4) | 1.00 (Referent) | | 1.00 (Referent) | |
| >4-7 | 131 (25.5) | 0.47 (0.21, 1.01) | | 1.28 (0.69, 2.38) | | >4-6 | 82 (21.5) | 0.20 (0.07, 0.54) | | 0.25 (0.09, 0.67) | |
| >7-12 | 109 (21.2) | 0.63 (0.29, 1.39) | | 0.93 (0.47, 1.86) | | >6-11 | 103 (27.0) | 0.27 (0.11, 0.64) | | 0.68 (0.31, 1.48) | |
| >12 | 142 (27.6) | 0.61 (0.30, 1.23) | | 1.00 (0.53, 1.86) | | >11 | 96 (25.1) | 0.21 (0.08, 0.54) | | 0.56 (0.25, 1.27) | |
| Dietary | | | 0.81 | | 0.98 | Dietary | | | 0.001 | | 0.11 |
| 0-3 | 104 (20.2) | 1.00 (Referent) | | 1.00 (Referent) | | 0-4 | 114 (29.7) | 1.00 (Referent) | | 1.00 (Referent) | |
| >3-5 | 150 (29.2) | 1.31 (0.59, 2.93) | | 0.92 (0.48, 1.75) | | >4-6 | 100 (26.0) | 0.27 (0.11, 0.65) | | 0.43 (0.19, 0.98) | |
| >5-7 | 127 (24.7) | 1.24 (0.53, 2.90) | | 0.98 (0.50, 1.90) | | >6-8 | 75 (19.5) | 0.47 (0.20, 1.08) | | 0.96 (0.44, 2.09) | |
| >7 | 133 (25.9) | 1.54 (0.65, 3.65) | | 0.88 (0.43, 1.79) | | >8 | 95 (24.7) | 0.13 (0.04, 0.39) | | 0.51 (0.22, 1.21) | |
| Supplements | | | 0.79 | | 0.90 | Supplements | | | 0.11 | | 0.55 |
| No | 346 (67.3) | 1.00 (Referent) | | 1.00 (Referent) | | No | 316 (82.3) | 1.00 (Referent) | | 1.00 (Referent) | |
| Yes | 168 (32.7) | 0.80 (0.45, 1.41) | | 0.97 (0.61, 1.55) | | Yes | 68 (17.7) | 0.45 (0.17, 1.20) | | 0.79 (0.38, 1.68) | |
| **Vitamin B2 (mg)** | | | 0.76 | | 0.76 | **Vitamin B2 (mg)** | | | 0.27 | | 0.11 |
| 0-2.2 | 148 (28.8) | 1.00 (Referent) | | 1.00 (Referent) | | 0-1.9 | 88 (23.1) | 1.00 (Referent) | | 1.00 (Referent) | |
| >2.2-3.0 | 115 (22.4) | 0.92 (0.44, 1.93) | | 0.78 (0.42, 1.48) | | >1.9-2.6 | 97 (25.5) | 0.60 (0.23, 1.57) | | 0.35 (0.14, 0.89) | |
| >3.0-4.0 | 129 (25.1) | 0.67 (0.31, 1.43) | | 0.83 (0.45, 1.54) | | >2.6-3.7 | 99 (26.0) | 1.28 (0.50, 3.23) | | 0.90 (0.39, 2.08) | |
| >4.0 | 122 (23.7) | 0.81 (0.36, 1.84) | | 0.69 (0.34, 1.40) | | >3.7 | 97 (25.5) | 0.65 (0.21, 2.01) | | 0.64 (0.24, 1.71) | |
| **Vitamin B6 (mg)** | | | 0.71 | | 0.79 | **Vitamin B6 (mg)** | | | 0.66 | | 0.37 |
| 0-2.0 | 133 (25.9) | 1.00 (Referent) | | 1.00 (Referent) | | 0-1.7 | 97 (25.4) | 1.00 (Referent) | | 1.00 (Referent) | |
| >2.0-2.7 | 116 (22.6) | 1.32 (0.62, 2.81) | | 1.03 (0.55, 1.94) | | >1.7-2.2 | 87 (22.8) | 0.65 (0.25, 1.65) | | 0.48 (0.19, 1.24) | |
| >2.7-4.2 | 134 (26.1) | 0.88 (0.40, 1.96) | | 0.81 (0.42, 1.56) | | >2.2-3.2 | 104 (27.2) | 0.96 (0.38, 2.43) | | 1.02 (0.44, 2.38) | |
| >4.2 | 131 (25.5) | 0.90 (0.40, 2.01) | | 0.77 (0.39, 1.52) | | >3.2 | 94 (24.6) | 0.65 (0.22, 1.88) | | 0.95 (0.38, 2.40) | |
| **Vitamin B12 (ug)** | | | 0.69 | | 0.41 | **Vitamin B12 (ug)** | | | 0.21 | | 0.91 |

| | | Ontario | | | | | Newfoundland | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *DKK1* | | *Wnt5a* | | | *DKK1* | | *Wnt5a* | |
| **Variable** | **Total (%)** | **OR (95% CI)** | **p** | **OR (95% CI)** | **p** | **Variable** | **Total (%)** | **OR (95% CI)** | **p** | **OR (95% CI)** | **p** |
| 0-5 | 124 (24.1) | 1.00 (Referent) | | 1.00 (Referent) | | 0-5 | 92 (24.1) | 1.00 (Referent) | | 1.00 (Referent) | |
| >5-8 | 133 (25.9) | 1.41 (0.66, 3.00) | | 1.48 (0.79, 2.77) | | >5-8 | 95 (24.9) | 0.43 (0.18, 1.04) | | 0.84 (0.37, 1.89) | |
| >8-16 | 126 (24.5) | 1.09 (0.48, 2.47) | | 0.94 (0.47, 1.90) | | >8-13 | 99 (25.9) | 0.51 (0.21, 1.24) | | 0.74 (0.32, 1.74) | |
| >16 | 131 (25.5) | 0.91 (0.42, 2.00) | | 0.94 (0.49, 1.81) | | >13 | 96 (25.1) | 0.47 (0.19, 1.21) | | 0.90 (0.38, 2.11) | |
| **Folate (ug)** | | | 0.74 | | 0.61 | **Folate (ug)** | | | 0.65 | | 0.73 |
| 0-550 | 130 (25.3) | 1.00 (Referent) | | 1.00 (Referent) | | 0-300 | 84 (22.0) | 1.00 (Referent) | | 1.00 (Referent) | |
| >550-840 | 129 (25.1) | 0.94 (0.45, 1.97) | | 0.69 (0.36, 1.30) | | >300-400 | 101 (26.5) | 0.94 (0.36, 2.44) | | 1.26 (0.50, 3.20) | |
| >840-1240 | 127 (24.7) | 0.68 (0.31, 1.49) | | 0.78 (0.41, 1.48) | | >400-525 | 101 (26.5) | 1.38 (0.52, 3.69) | | 1.68 (0.65, 4.30) | |
| >1240 | 128 (24.9) | 0.73 (0.32, 1.65) | | 0.66 (0.33, 1.31) | | >525 | 95 (24.9) | 0.83 (0.23, 2.99) | | 1.36 (0.43, 4.29) | |
| **Isoflavonoids (mg)** | | | 0.64 | | 0.15 | **Isoflavonoids (mg)** | | | 0.21 | | 0.13 |
| 0-0.3 | 113 (22.0) | 1.00 (Referent) | | 1.00 (Referent) | | 0-0.1 | 106 (27.6) | 1.00 (Referent) | | 1.00 (Referent) | |
| >0.3-0.5 | 123 (23.9) | 1.03 (0.49, 2.16) | | 0.48 (0.25, 0.93) | | >0.1-0.25 | 91 (23.7) | 1.18 (0.44, 3.16) | | 2.00 (0.82, 4.90) | |
| >0.5-0.8 | 136 (26.5) | 0.91 (0.43, 1.92) | | 0.61 (0.32, 1.15) | | >0.25-0.32 | 88 (22.9) | 1.99 (0.80, 4.92) | | 2.52 (1.05, 6.07) | |
| >0.8 | 142 (27.6) | 0.62 (0.27, 1.47) | | 0.77 (0.40, 1.49) | | >0.32 | 99 (25.8) | 2.23 (0.93, 5.33) | | 2.65 (1.13, 6.20) | |
| **Fibre (g)** | | | 0.37 | | 0.18 | **Fibre (g)** | | | 0.53 | | 0.62 |
| 0-18 | 124 (24.1) | 1.00 (Referent) | | 1.00 (Referent) | | 0-15 | 101 (26.3) | 1.00 (Referent) | | 1.00 (Referent) | |
| >18-24 | 131 (25.5) | 1.50 (0.71, 3.13) | | 0.75 (0.41, 1.39) | | >15-20 | 81 (21.1) | 1.52 (0.62, 3.69) | | 1.22 (0.51, 2.92) | |
| >24-32 | 126 (24.5) | 0.83 (0.36, 1.91) | | 0.46 (0.23, 0.92) | | >20-27 | 99 (25.8) | 0.80 (0.30, 2.12) | | 1.02 (0.42, 2.92) | |
| >32 | 133 (25.9) | 0.84 (0.33, 2.12) | | 0.68 (0.32, 1.43) | | >27 | 103 (26.8) | 1.15 (0.39, 3.38) | | 1.52 (0.58, 4.00) | |
| **Fat** | | | | | | **Fat** | | | | | |
| Daily Intake (g) | | | 0.31 | | 0.39 | Daily Intake (g) | | | 0.13 | | 0.41 |
| 0-60 | 146 (28.4) | 1.00 (Referent) | | 1.00 (Referent) | | 0-60 | 103 (26.8) | 1.00 (Referent) | | 1.00 (Referent) | |
| >60-80 | 129 (25.1) | 1.65 (0.77, 3.55) | | 1.71 (0.92, 3.16) | | >60-80 | 90 (23.4) | 0.33 (0.12, 0.92) | | 0.51 (0.21, 1.26 | |
| >80-100 | 102 (19.8) | 1.87 (0.76, 4.58) | | 1.34 (0.64, 2.78) | | >80-110 | 100 (26.0) | 0.89 (0.37, 2.13) | | 0.93 (0.40, 2.15) | |
| >100 | 137 (26.7) | 2.93 (0.95, 9.02) | | 1.36 (0.55, 3.36) | | >110 | 91 (23.7) | 0.48 (0.12, 1.92) | | 1.06 (0.32, 3.46) | |
| % Daily Calories | | | 0.30 | | 0.25 | % Daily Calories | | | 0.81 | | 0.40 |
| 0-28 | 142 (27.6) | 1.00 (Referent) | | 1.00 (Referent) | | 0-27 | 93 (24.2) | 1.00 (Referent) | | 1.00 (Referent) | |
| >28-32 | 108 (21.0) | 1.78 (0.82, 3.89) | | 1.39 (0.75, 2.60) | | >27-31 | 91 (23.7) | 0.81 (0.35, 1.87) | | 0.88 (0.42, 1.86) | |
| >32-36 | 134 (26.1) | 1.79 (0.85, 3.76) | | 0.88 (0.47, 1.65) | | >31-35 | 108 (28.1) | 0.78 (0.35, 1.75) | | 0.54 (0.25, 1.16) | |

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| | | Ontario | | | | | | Newfoundland | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *DKK1* | | *Wnt5a* | | | | *DKK1* | | *Wnt5a* | |
| **Variable** | **Total (%)** | **OR (95% CI)** | **p** | **OR (95% CI)** | **p** | **Variable** | **Total (%)** | **OR (95% CI)** | **p** | **OR (95% CI)** | **p** |
| >36 | 130 (25.3) | 1.94 (0.90, 4.18) | | 1.54 (0.84, 2.95) | | >35 | 92 (24.0) | 0.64 (0.26, 1.56) | | 0.67 (0.30, 1.49) | |
| **Saturated Fat** | | | | | | **Saturated Fat** | | | | | |
| Daily Intake (g) | | | 0.48 | | 0.16 | Daily Intake (g) | | | 0.72 | | 0.86 |
| 0-20 | 146 (28.4) | 1.00 (Referent) | | 1.00 (Referent) | | 0-20 | 98 (25.5) | 1.00 (Referent) | | 1.00 (Referent) | |
| >20-25 | 104 (20.2) | 1.65 (0.75, 3.62) | | 0.99 (0.50, 1.98) | | >20-28 | 108 (28.1) | 0.64 (0.28, 1.48) | | 0.87 (0.40, 1.91) | |
| >25-35 | 147 (28.6) | 1.68 (0.77, 3.68) | | 1.84 (0.98, 3.46) | | >28-38 | 81 (21.1) | 0.64 (0.24, 1.72) | | 0.80 (0.32, 1.98) | |
| >35 | 117 (22.8) | 2.30 (0.73, 7.28) | | 1.37 (0.53, 3.54) | | >38 | 97 (25.3) | 0.57 (0.16, 2.00) | | 0.60 (0.18, 1.93) | |
| % Daily Calories | | | 0.32 | | 0.03 | % Daily Calories | | | 0.63 | | 0.12 |
| 0-9.0 | 130 (25.3) | 1.00 (Referent) | | 1.00 (Referent) | | 0-9 | 96 (25.0) | 1.00 (Referent) | | 1.00 (Referent) | |
| >9.0-10.5 | 114 (22.2) | 1.17 (0.52, 2.65) | | 0.96 (0.47, 1.98) | | >9-11 | 113 (29.4) | 0.70 (0.32, 1.54) | | 0.71 (0.36, 1.43) | |
| >10.5-12.0 | 135 (26.3) | 1.43 (0.67, 3.03) | | 2.05 (1.10, 3.84) | | >11-13 | 98 (25.5) | 0.60 (0.26, 1.38) | | 0.40 (0.18, 0.89) | |
| >12.0 | 135 (26.3) | 1.94 (0.93, 4.06) | | 1.96 (1.04, 3.70) | | >13 | 77 (20.1) | 0.66 (0.26, 1.65) | | 0.50 (0.21, 1.17) | |
| **Cholesterol (mg)** | | | 0.25 | | 0.30 | **Cholesterol (mg)** | | | 0.50 | | 0.61 |
| 0-180 | 111 (21.6) | 1.00 (Referent) | | 1.00 (Referent) | | 0-225 | 102 (26.6) | 1.00 (Referent) | | 1.00 (Referent) | |
| >180-260 | 151 (29.4) | 1.94 (0.92, 4.09) | | 1.31 (0.69, 2.48) | | >225-300 | 93 (24.2) | 0.72 (0.32, 1.63) | | 0.71 (0.33, 1.56) | |
| >260-340 | 130 (25.3) | 1.34 (0.57, 3.13) | | 1.61 (0.81, 3.19) | | >300-400 | 81 (21.1) | 0.60 (0.24, 1.52) | | 0.60 (0.25, 1.42) | |
| >340 | 122 (23.7) | 1.05 (0.35, 3.12) | | 0.91 (0.37, 2.23) | | >400 | 108 (28.1) | 0.43 (0.14, 1.30) | | 0.55 (0.21, 1.46) | |

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**Table 3**

Associations between lifestyle factors and methylation status of *DKK1* and *Wnt5a* in colorectal carcinomas from Ontario and Newfoundland. Models were adjusted for age, sex, and total energy intake.

| | | Ontario | | | | | | Newfoundland | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *DKK1* | | *Wnt5a* | | | | *DKK1* | | *Wnt5a* | |
| **Variable** | **Total (%)** | **OR (95% CI)** | **p** | **OR (95% CI)** | **p** | **Variable** | **Total (%)** | **OR (95% CI)** | **p** | **OR (95% CI)** | **p** |
| **Alcohol** | | | | | | **Alcohol** | | | | | |
| Daily Intake (g) | | | 0.16 | | 0.02 | Daily Intake (g) | | | 0.98 | | 0.89 |
| 0 | 136 (26.4) | 1.00 (Referent) | | 1.00 (Referent) | | 0 | 206 (51.6) | 1.00 (Referent) | | 1.00 (Referent) | |
| >0-11 | 205 (39.7) | 0.59 (0.31, 1.10) | | 0.45 (0.26, 0.77) | | >0-9 | 74 (18.5) | 0.94 (0.41, 2.17) | | 0.98 (0.46, 2.12) | |
| >11 | 175 (33.9) | 0.56 (0.27, 1.14) | | 0.60 (0.33, 1.09) | | >9 | 119 (29.8) | 0.92 (0.42, 2.01) | | 0.85 (0.42, 1.70) | |
| % Daily Calories | | | 0.34 | | 0.85 | % Daily Calories | | | 0.85 | | 0.84 |
| 0-0.5 | 188 (36.6) | 1.00 (Referent) | | 1.00 (Referent) | | 0 | 143 (37.2) | 1.00 (Referent) | | 1.00 (Referent) | |
| >0.5-4.5 | 169 (32.9) | 1.12 (0.61, 2.04) | | 1.00 (0.59, 1.72) | | >0-3 | 124 (32.3) | 0.91 (0.45, 1.82) | | 0.98 (0.52, 1.87) | |
| >4.5 | 157 (30.5) | 0.65 (0.31, 1.33) | | 1.16 (0.66, 2.05) | | >3 | 117 (30.5) | 0.79 (0.35, 1.80) | | 0.81 (0.39, 1.69) | |
| **Smoking** | | | 0.83 | | 0.04 | **Smoking** | | | 0.88 | | 0.12 |
| No | 190 (37.0) | 1.00 (Referent) | | 1.00 (Referent) | | No | 115 (28.0) | 1.00 (Referent) | | 1.00 (Referent) | |
| Yes | 324 (63.0) | 1.10 (0.63, 1.92) | | 0.62 (0.39, 0.98) | | Yes | 296 (72.0) | 0.95 (0.49, 1.84) | | 0.63 (0.35, 1.13) | |
| **BMI** | | | | | | **BMI** | | | | | |
| Current | | | 0.05 | | 0.62 | Current | | | 0.45 | | 0.26 |
| <25 | 175 (36.2) | 1.00 (Referent) | | 1.00 (Referent) | | ≤5 | 118 (29.6) | 1.00 (Referent) | | 1.00 (Referent) | |
| >25-30 | 192 (39.7) | 0.44 (0.23, 0.87) | | 0.81 (0.47, 1.39) | | >25-30 | 162 (40.6) | 0.87 (0.43, 1.79) | | 0.57 (0.29, 1.12) | |
| >30 | 117 (24.2) | 0.88 (0.45, 1.75) | | 1.07 (0.59, 1.94) | | >30 | 119 (29.8) | 0.59 (0.25, 1.36) | | 0.78 (0.38, 1.60) | |
| Age 20 | | | 0.76 | | 0.02 | Age 20 | | | 0.94 | | 0.99 |
| <25 | 354 (74.4) | 1.00 (Referent) | | 1.00 (Referent) | | <25 | 307 (79.3) | 1.00 (Referent) | | 1.00 (Referent) | |
| >25 | 122 (25.6) | 1.11 (0.57, 2.17) | | 1.93 (1.12, 3.31) | | >25 | 80 (20.7) | 1.03 (0.44, 2.41) | | 1.01 (0.46, 2.19) | |