INFLUENCE OF ENVIRONMENTAL RISK FACTORS OF COLORECTAL CANCER AND GENE-ENVIRONMENT INTERACTIONS ON LINE-1 DNA METHYLATION

by

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Abstract

Introduction: This study examined the association between established colorectal cancer (CRC) risk factors and LINE-1 DNA methylation in healthy colon tissue, and whether genes involved in one-carbon metabolism modify relationships between established CRC risk factors and LINE-1 DNA methylation. This study was undertaken in order to inform on the role of LINE-1 DNA methylation as a mechanism by which established risk factors may lead to carcinogenesis. Reduced LINE-1 DNA methylation and one-carbon metabolism-related gene variants are both shown to increase CRC risk. Exposures related to LINE-1 DNA methylation may have a greater effect on methylation levels for individuals already susceptible to reduced methylation due to possession of a genetic variant. When considered together, these effects may impact methylation patterns in individuals beyond what would be expected if effects were purely additive.

Methods: The study population consisted of 317 individuals scheduled for a routine colonoscopy. Multivariable linear regression was used to study the associations of alcohol consumption, smoking, body mass index, non-steroidal anti-inflammatory drug use, physical activity, and fruit and vegetable consumption with LINE-1 DNA methylation. Product terms between *MTHFR* 677C>T (rs1801133), *MTR* 2756A>G (rs1805087), and *MTRR* 66A>G (rs1801394) genotypes and exposures of interest were used to examine effect modification by genotype.

Results: There were no statistically significant relationships observed between each risk factor of interest and LINE-1 DNA methylation. Statistically significant interactions between alcohol consumption and *MTHFR* and *MTR* genotypes were observed (p-value interaction=0.009, 0.003) in relation to LINE-1 DNA methylation.

Conclusion: This thesis presents novel results on the relationships between a comprehensive set of CRC risk factors and LINE-1 DNA methylation in healthy colon tissue. We observed significant interactions by gene variants in the relationships between CRC risk factors and LINE-1 DNA methylation in the healthy colon. Although effects were not consistently in hypothesized directions, they suggest that one-carbon metabolism-related gene status is an important effect modifier in the relationship between alcohol consumption and LINE-1 DNA methylation.

Co-Authorship

The conceptualization of this thesis project, the review of the literature, and the cleaning, linkage, and analysis of the data, as well as the preparation of the two manuscripts was done by Priyanka Gogna. Will King provided feedback at every stage of the thesis regarding the design of the study, the creation of the variables, analysis, and interpretation of the data.

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List of Abbreviations

ANOVA – Analysis of variance

BMI – Body mass index

CpG – Cytosine phosphate guanine

CRC – Colorectal cancer

DNMT – DNA methyltransferase

HRMC – High-resolution melt-curve

IPAQ-SF – International physical activity questionnaire-short form

L1 – Long interspersed nuclear element 1, LINE- 1

MAF – Minor allele frequency

MET – Metabolic equivalent of task

NSAID – Non-steroidal anti-inflammatory drug

PBL – Peripheral blood leukocyte

PCR – polymerase chain reaction

SAM – S-adenosylmethionine

SINEs – Short interspersed nuclear elements

Chapter 1

Introduction

After sequencing of the human genome in 2003, science turned to the epigenome to improve understanding of the biological processes that dictate how the genetic code is read (1,2). The epigenome is a set of heritable, yet modifiable chemical changes that affect the transcription and translation of the genome (2). The pattern of the epigenome is responsible for gene activation, silencing, and facilitation of tissue-specific gene expression in cells of the human body. Although our genetic code is fixed, the human epigenome is susceptible to change due to the environment, and can vary across an individual's lifetime (3). This key characteristic has made the epigenome an important research topic in human genetics and health sciences (1,4). DNA methylation, histone modification, nucleosome modeling, and miRNAs are known epigenetic mechanisms (1).

DNA methylation is a widely studied epigenetic modification in the field of molecular epidemiology, which seeks to understand molecular processes by which known risk factors may cause disease in the human body. Molecular epidemiology techniques are commonly applied to research focused on the etiology of cancer, where long latency periods make it difficult to study the effect of toxins on cancer risk. By breaking down the disease continuum into molecular pathways and relationships, molecular epidemiologists can elaborate on the processes by which lifestyle exposures can lead to disease. This field of study relies on continuing research by basic scientists and traditional epidemiology researchers to understand biological processes, as well as relationships between risk factors and outcomes.

Another consideration of molecular epidemiology research involves the investigation of genetic factors that affect the molecular processes under study. The efficiency of molecular processes in the cell is influenced by an individual's genetics (5). By incorporating genetic factors into the study of molecular epidemiology, it becomes possible to target those individuals that are at an increased risk of disease due to genetic polymorphisms. By combining data on lifestyle exposures and genetic factors, a more complete picture of chronic disease risk begins to form. This research can then be directly used to test individuals for genetic variants, as well as create targeted interventions for those at an increased risk. More generally, molecular epidemiology allows intervention research to move forward with greater information regarding the biological mechanisms behind disease causation (6). Disease understanding is arguably the first step to towards disease reduction.

This master's thesis is guided by a molecular epidemiology approach. Using a comprehensive set of known colorectal cancer risk factors, including alcohol consumption, smoking, obesity, non-steroidal anti-inflammatory drug use, physical activity, and fruit and vegetable consumption, I sought to understand the mechanistic role of Long interspersed nuclear element-1 (L1) DNA methylation in the healthy colon as a process by which these risk factors may lead to an increased risk of colorectal adenomatous polyps (adenoma). Although the role of L1 DNA methylation in cancer development has stimulated considerable research in the past years, the majority of this research uses convenience samples taken from blood instead of using healthy colon tissue (6) (7) (8-15). Studies that have examined methylation in blood are not ideal as blood is not the target tissue of interest, and methylation measures in blood only moderately

correlate with measures in colon tissue (16). Studies focusing on adenoma or CRC tissue are also inappropriate as aberrant methylation in this tissue may be a consequence, rather than a cause of disease. The use of samples from the tissue of interest present a novel aspect of this study in comparison to previous analyses.

The original proposal for this work included mediation analysis in order to formally assess the role of L1 DNA methylation as a mediator of the relationship between each lifestyle exposure and L1 DNA methylation. However, relationships between variables did not allow for completion of this objective. This manuscript-based thesis begins with a chapter presenting a systematic literature review of current research on the relationships of interest, and a chapter on the original study design and analyses done prior to manuscript preparation. The first manuscript chapter investigates the main effects of each risk factor in relation to L1 DNA methylation, and the second assesses potential effect modification by gene variants known to influence L1 DNA methylation. The discussion chapter further elaborates on strengths and limitations of this project, as well as study implications and directions for future research.

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Chapter 2

Background and Literature Review

2.1 Introduction to Epigenetics and DNA Methylation

Epigenetic processes represent an important aspect of cancer etiology research focusing on how environmental processes influence the epigenome. This relatively new field presents an exciting and hopeful focus on how we can modify genetic processes and potentially change the "genetic hand" we are dealt at birth. Without changing the underlying sequence, epigenetic alterations are able to affect expression of the genome, and pass these changes on from one cell to another (1). Furthermore, epidemiologic investigation of lifestyle, occupational and environmental risk factors in relation to epigenetic markers has provided evidence that the epigenome is modifiable (2-4).

The application of epigenetic research is easily seen in cancer epidemiology: instead of focusing on non-modifiable risk factors such as rare gene variants, epigenetic research provides information on how modifiable mechanisms impact cancer risk, which can be used to directly inform endeavors in cancer prevention.

While several known epigenetic modifications have been discovered in recent years, DNA methylation is the most commonly studied modification in relation to cancer risk, and is recognized as an important factor in tissue-specific gene expression, genomic stability, and human disease (1).

2.2 Mechanisms of DNA Methylation

DNA methylation occurs when a cytosine nucleotide within a cytosine-phosphate-guanine (CpG) site on a DNA strand becomes methylated to produce 5-methylcytosine

(5). This modification impedes further transcription of the DNA section by affecting interactions with transcriptional proteins in the nucleus, allowing methylation to control transcriptional processes by down-regulating gene expression. Repeat-rich regions of the genome are often methylated, while the promoter regions of a specific gene are often not (6). Aberrant methylation of the genome occurs when methylation patterns deviate from what would be expected in that region of genome (6).

DNA methylation patterns are controlled in the cell by DNA methyltransferases (DNMTs). These enzymes are responsible for *de novo* methylation, as well as methylation addition, removal, or maintenance on the DNA molecule (7). Specifically, DNMTs are essential to the carrying over of methylation patterns from parent cells to daughter cells, as well as any modification that may occur to both gene-specific and repetitive element methylation across a lifetime. The efficiency of the methylation process itself is dependent on one-carbon metabolism, as this cellular process donates the methyl group required by DNMTs to facilitate methylation (7). Therefore, suspected lifestyle and environmental risk factors that disrupt one carbon metabolism may impact cancer risk via DNA methylation mechanisms (8).

Hypermethylation of DNA is often discussed in the context of the down-regulation, or "turning off" of key tumour suppressor genes via increases in 5-methylcytosines in the promoter regions of CpG islands (9). These tumour-suppressor genes are involved in DNA repair, the cell cycle, and apoptosis. Down-regulation of genes involved in important metabolic processes, detoxification, or oxidative stress also represents important hypermethylation activity linked to carcinogenesis (10).

In contrast to gene-specific methylation, repetitive elements are typically highly methylated. Hypomethylation of repetitive elements affects chromosomal stability, and is thought to occur prior to hypermethylation of gene-specific promoter regions (11,12).

Methylation patterns are relatively stable across short time periods, such as month-to-month, or even year-to-year (13-16). However, genome-wide methylation levels are known to vary based on age, sex, race, and genetic polymorphisms, and may be modified across longer timescales due to environmental and lifestyle factors (2-4). Aberrant changes in methylation are concerning, and have been linked to cancer, as well as several other disease processes in humans (9).

2.3 LINE-1 DNA Methylation

Repetitive elements are elements across the genome with repeating base pairs. These elements make up approximately half of the human genome (17). The most common sub-classification of these elements is interspersed repetitive elements. Short interspersed nuclear elements (SINES) are less than 500 base pairs long, while long interspersed nuclear elements (LINEs) can be longer than 5000 base pairs. LINE-1 (L1) elements are the most common form of LINEs, with over 500,000 copies in the genome, and Alu elements are the most common form of SINEs, with over 1,000,000 copies (17). L1 repeats make up approximately 20% of the human genome, and SINEs comprise approximately 15% of the genome (17). Both interspersed repetitive elements have the ability to move and amplify within a genome, and contain considerably larger numbers of CpG sites than other regions of the genome (18). Under normal conditions, CpG sites within these elements are highly methylated, and play a direct role in silencing and stabilizing these elements (19).

Due to the extensive nature of L1 elements in the genome, L1 DNA methylation has been considered a proxy for global DNA methylation across the genome. Therefore, discussion of global methylation in the literature is strongly tied to the study of L1 DNA methylation, despite the fact that L1 DNA methylation is a specific, and measurable effect, while global methylation vaguely refers to any or all methylation occurring outside of genes (20). Discussion of both global methylation and L1 DNA methylation is distinct from gene-specific changes in methylation. Aberrant L1 DNA methylation in both contexts has been linked to an increase in various diseases, including cancer (9,21).

2.3.1 Determinants of LINE-1 DNA Methylation

L1 DNA methylation is a modifiable epigenetic mechanism that has been shown to remain stable over shorter time-periods, while potentially being impacted by environmental exposures over longer time periods (15,16,22). Specifically, several non-modifiable factors are known to affect L1 DNA methylation throughout life. Age is perhaps the most well studied factor related to L1 DNA methylation, to the extent that epigenetic measures have been used as "clocks" to measure the biological age of tissue (23). A negative relationship between age and L1 DNA methylation is documented in the literature, making age an important confounding variable in any study addressing L1 DNA methylation as an outcome (24).

L1 DNA methylation levels differ between men and women in epidemiological studies. Typically, women tend to have lower L1 methylation levels than men (2,25,26). Although the mechanism leading to differential L1 DNA methylation between men and women is not yet clear, sex is an important variable for analysis in DNA methylation research.

Although ethnicity has the propensity to affect L1 DNA methylation patterns through its direct association with genetic background, there is a lack of evidence supporting a relationship between ethnicity and DNA methylation (2,3).

Folate and B_{12} are two dietary vitamins that are strongly linked to DNA methylation in the cell through their direct involvement in molecular processes essential to DNA methylation (27,28). Although they are often studied under the context of dietary factors influencing L1 DNA methylation, their strong relationship with DNA methylation requires their consideration in all studies focusing on L1 DNA methylation.

Therefore, age, sex, ethnicity, folate, and B_{12} have the potential to affect DNA methylation patterns and should be considered potential confounders in the analysis of modifiable lifestyle factors and L1 DNA methylation.

2.4 LINE-1 DNA Methylation and Cancer

Feinberg et al. first examined the relationship between DNA methylation and cancer in 1983 (29). Since this initial discovery, a substantial body of evidence establishing the association between both aberrant methylation patterns has emerged in the medical literature (8,9,19,30,31). Both hypermethylation of tumour-suppressor and regulatory genes, and hypomethylation of non-coding DNA have been linked via separate biological mechanisms to a higher risk of cancer (32).

Hypermethylation involves the “turning off” of key tumour suppressor genes via increases in 5-methylcytosines in the promoter regions of CpG islands (9). These tumour-suppressor genes are involved in a number of important cellular tasks, such as DNA repair, the cell cycle, and apoptosis. Increased methylation of these genes is considered a precursor to many cancers such as breast, colorectal, stomach, and esophageal cancer (9).

In contrast to gene-specific methylation, repetitive elements are typically highly methylated. Hypomethylation of these elements affects chromosomal stability, and is known to occur prior to hypermethylation in gene-specific promoter regions (11,12). Hypomethylation of repetitive regions of DNA such as L1 repeat regions has been linked to colorectal, lung, bladder, kidney, and liver cancers, among others (9).

2.5 LINE-1 DNA Methylation as a Biomarker of Early Carcinogenic Effect

Biomarkers have become commonplace surrogate endpoints in clinical research design due to ease of measurement and earlier detection of disease (33). When measured accurately and reproducibly, they can provide objective indications of medical state in a patient (33).

L1 DNA methylation is an early carcinogenic event, and has been specifically linked to colorectal, breast, cervical, head and neck, and kidney cancer, as well as all-cancer mortality, and other chronic diseases (16,34,35). Furthermore, L1 DNA methylation is a relatively stable measure, and does not change over shorter time periods. Although prospective studies on DNA methylation status are limited, an intervention study found no change in L1 DNA methylation 24 hours after a vigorous exercise intervention in athletes (14). Another methods-based paper found no significant changes in global methylation at 10 months, and ten months plus 1 week (13). Other studies have also found that DNA methylation is tightly regulated with stable measures over 12 months (15,16). Stability is an essential component of a biomarker, as a measure with poor stability and daily or monthly fluctuations would be difficult to measure without consistent measurement and follow-up.

L1 DNA methylation is also shown to lie on the causal pathway between certain lifestyle exposures and carcinogenesis, and basic science studies confirm that there is biological plausibility for L1 DNA methylation to lie on the causal pathway between exposure and disease (36-38).

Methods of quantifying L1 DNA methylation are complex, but recent technological advances have allowed for more precise measurement with reduced processing costs (39,40). It is perhaps these future advancements in cheaper, easier to use measurement tools that will further propel the use of L1 DNA methylation as a common biomarker to detect carcinogenesis.

Combined, these aspects of L1 DNA methylation demonstrate its potential as a biomarker of carcinogenesis, and suggest that investigating L1 DNA methylation as an outcome may be used to further study its role as a biological mechanism by which lifestyle factors may lead to an increased risk of cancer.

2.6 Analyzing DNA Methylation Levels in Human Tissues

Despite its potential as an early biomarker for cancer risk, several considerations must be made in analyses investigating L1 DNA methylation as an outcome. Methylation is a difficult biomarker to measure, both due to differing measurement techniques and differential methylation patterns expected across tissues. DNA methylation levels are assessed via several different methods aimed at quantifying the amount of methylated cytosine content present in samples. Liquid chromatography, enzyme-linked immunosorbent assay based methods, and restriction-fragment-length polymorphisms are some common methods through which L1 DNA methylation has been quantified in the literature (39). These methods have been tested for reliability, and are commonly used for

quantification of L1 DNA methylation, leading to substantial heterogeneity in methods used in molecular epidemiology studies focusing on L1 DNA methylation (39).

Unfortunately, due to variation in cost and genomic science expertise required to apply specific methods, no universal method has been adapted. This hinders the ability to discuss effect sizes for the relationships between lifestyle factors and L1 DNA methylation. For example, different laboratory methods may lead to overall higher or lower methylation measurement (39). Furthermore, previous studies of L1 DNA methylation quantify methylation differently, such as by incorporating SINE and LINE methylation, referring to methylation measurement as "global", and use of [^{3}H]methyl incorporation (an increase in [^{3}H]methyl incorporation reflects a decrease in DNA methylation), to name a few (41,42,43,44).

More recently, microchip-based approaches have been used to quantify DNA methylation. If adapted universally, this method may help standardized methylation measurement. Microchip-based approaches are able to quantify methylation across larger areas of the genome, however, they do not necessarily capture a higher number of L1 repeats than PCR-based techniques in which primers are specifically designed to quantify repetitive element methylation (40).

2.7 Analyses in Target Tissues

Peripheral blood leukocytes (PBLs) are a popular tissue of choice for the analysis of DNA methylation, as it is a convenient source of DNA. However, DNA methylation patterns are tissue-specific, and methylation patterns found in PBLs may not reflect methylation occurring in tissues of interest, such as colon mucosal tissue. Analysis of L1 DNA methylation in PBLs and colon mucosal tissue samples reveal weak correlations in

methylation patterns between the two tissues (r=0.36) (45). Although comparison of L1 levels in PBLs and colon tissue is limited, similar analyses focusing on blood and kidney tissues also report low correlation values (r=0.08) (46). This finding suggests caution when comparing results from methylation analysis of PBLs, and emphasizes the importance of methylation measurement directly from tissues of interest. Furthermore, recent research suggests that variability in DNA methylation found in PBLs may be a function of the type of blood cells chosen for analysis, rather than person-to-person variability due to lifestyle factors, and although this concern may remain in regard to cell type distributions in tissues of interest, it may be mitigated depending on the type of tissue, and sampling procedure (47).

Differences in DNA methylation measures, as well as discrepancies between the tissue analyzed and tissue of interest are two major sources of heterogeneity in the literature. Specifically, even once a target tissue is selected, differences in cell-type distribution across samples have the propensity to lead to misclassification of the outcome. Furthermore, risk factors of interest may also impact cell-type distributions themselves, making unmeasured confounding a potential problem in analyses. These differing measurement and analysis techniques present difficulties when comparing DNA methylation across studies, and may explain some discrepancies in directionality and magnitude of effects observed in the literature. However, despite these limitations, some of the discussed shortcomings may be overcome with careful study design and analyses.

2.8 Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer diagnosis worldwide, and is a cause of significant morbidity and mortality in North America (48). Colorectal

tumour development follows an established sequence from aberrant proliferative epithelial dysplasia, to colorectal adenomatous polyps (adenoma), to CRC (49). Adenomas are a recognized precursor in the pathogenesis of CRC. Although all adenomas do not result in CRC, most cases of CRC arise from adenomas in the colon (50). Approximately 50% of the general population will develop an adenoma in their lifetime (38).

2.8.1 Biological Mechanisms Relating LINE-1 DNA Methylation and CRC

Genome-wide hypomethylation has been linked with chromosomal instability and microsatellite instability, and these cellular events represent two forms of genomic instability by which L1 DNA methylation may cause CRC (36-38). Chromosomal instability refers to generalized chromosomal-level instability, and has been linked to chromosome deletions and duplications (aneuploidy) in the cell, conditions that are strongly linked to tumorigenesis (38). Microsatellite instability is a form of hyper-mutability of the genome that arises due to point-mutations leading to faulty DNA mismatch repair mechanisms (38). Although these mechanisms have been studied in colon tumour tissue, there is limited information on this relationship in healthy colon mucosal tissue.

2.9 Risk Factors for Colorectal Cancer and Association with LINE-1 DNA Methylation

The association between exposures including alcohol consumption, smoking, obesity, non-steroidal anti-inflammatory (NSAID) use, body mass index (BMI), diet, and CRC are established (12,51-60). However, mechanisms underlying these relationships are

Table 2-1 Relationships between lifestyle factors of interest and colorectal cancer risk

Lifestyle Exposure	Associated Risk Estimate for Colorectal Cancer (95% CI)*
Alcohol Consumption	1.13 (1.09-1.17) (61)
Smoking	1.25 (1.14-1.37) (62)
Obese BMI	1.12 (1.07-1.18) (63)
NSAID Use	0.78 (0.68-0.92) (64)
Physical Activity	0.63 (0.47-0.84) (65)
Fruit and Vegetable Consumption	0.82 (0.75-0.91) (66)

*Associated risks from recent meta-analyses for all exposures except for NSAID use. Risks represent comparisons of highest levels of exposure compared to respective referent categories for each lifestyle factor.
BMI-Body Mass Index
NSAID-Non-steroidal anti-inflammatory drug

not well understood, and L1 DNA methylation represents a potential mechanism of action behind the carcinogenic effects of these exposures. Furthermore, genetic variants may affect the relationship between these risk factors and DNA methylation. Previous research on biologically plausible mechanisms relating the listed exposures of interest to L1 DNA methylation supports further study of these relationships.

2.9.1 Biological Mechanisms

Biological mechanisms that support the relationships under study are complex, and associated with one another. One-carbon metabolism and inflammation represent two major mechanisms through which exposures of interest may be linked to aberrant L1 DNA methylation, and some risk factors may jointly affect both processes.

One-carbon metabolism comprises of a group of biochemical reactions at the cellular level that are involved in amino acid and nucleotide metabolism (67). One-carbon metabolism is specifically required for the addition of methyl groups to CpG sites in order for DNA to become methylated. Methyltransferases facilitate biochemical reactions involved, and use S-adenosylmethionine (SAM) as a methyl donor group.

However, methytransferases require tetrahydrofolate, a form of folate, as well as vitamin B_{12} as a co-enzyme for the reaction (68). Once SAM is activated to form methionine, the chemical is used for various cellular reactions, including DNA methylation (67).

Alcohol consumption is mechanistically linked to disruptions in the availability of folate in the body through intestinal malabsorption of folate, inhibition of important enzymes involved in one-carbon metabolism reactions, and the cleavage of folate by alcohol metabolites (67).

Smoking is known to affect DNA methylation, and one-carbon metabolism is a likely mechanism behind this relationship. Smoking can lead to hypoxia, which decreases oxygenation in the cell. Hypoxia directly affects methionine adenosyltransferase, which synthesizes SAM (69). This is a mechanism by which smoking can directly disrupt one-carbon metabolism and lead to aberrant L1 DNA methylation.

Inflammation is a biological response from the body's immune system in reaction to harmful stimuli. Despite its role as a key immune response, chronic inflammation can be harmful to the human body, and has been linked to L1 DNA methylation through several mechanisms (42). IL-6, a pro-inflammatory cytokine has been shown to directly affect genome-wide DNA hypomethylation (70). In addition, inflammation is responsible for the excess production of reactive oxygen species (71). This excess production is linked to deletions, base modifications, chromosomal rearrangements, and strand breakage in the DNA (71). Increased levels of reactive oxidative species cause oxidative stress in the cell, and can directly impact DNA methyltransferase activity as well. Prostaglandins are active lipid compounds that are also involved in inflammation, and also affect DNA methyltransferase activity. However, several of these studies have only

addressed their effect on gene-specific hypermethylation, and their effect on L1 DNA methylation requires further research (72,73).

Dietary habits such as high consumption of fruits and vegetables is linked to reduced inflammation in the literature, and obesity is an established cause of chronic inflammation in the health sciences (74-76). Physical exercise and NSAID use may play a protective role against chronic inflammation (42,44). The mechanisms discussed provide plausibility for these risk factors to affect L1 DNA methylation patterns in humans.

In conclusion, there exists a body of literature presenting biological mechanisms through which CRC risk factors may be associated with L1 DNA methylation patterns. These associations are complex, and likely interconnected at the molecular level, and risk factors such as alcohol consumption, smoking, and diet may mutually affect both one-carbon metabolism and inflammation (77-79). The conceptual model below depicts these relationships (Figure 2-1).

2.10 Systematic Literature Review: CRC Risk Factors and LINE-1 DNA methylation in the Healthy Colon

For the purposes of conducting a thorough and methodological appraisal of current literature on the relationships under study for this thesis, a systematic literature review was undertaken. The purpose of this search was to identify all current research on each of the relationships of interest with analysis conducted using healthy colon tissue. This search was updated March 13th, 2018. Due to the previously limitations of comparing methylation effect sizes in the literature, this review focuses on the statistical significance of relationships.

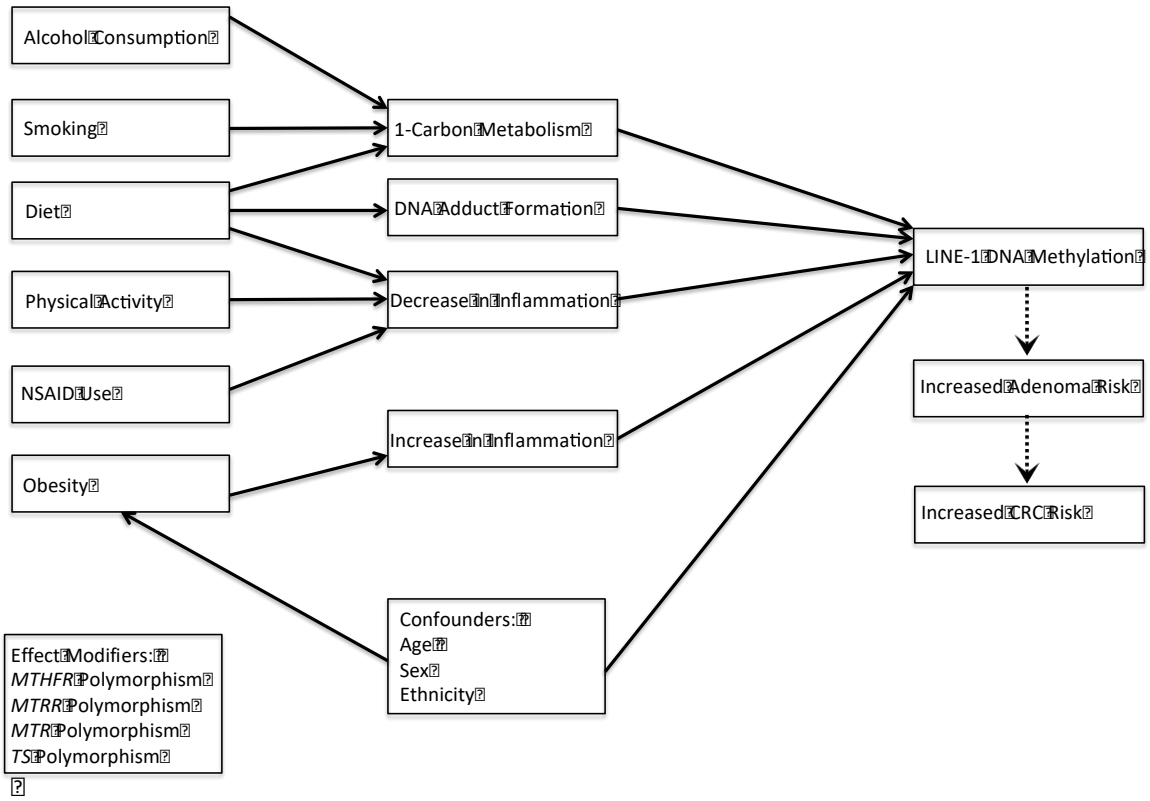

Figure 2-1. Biological mechanisms relating each exposure of interest to LINE-1 DNA methylation

2.10.1 Methods

Using advanced search term builders in both PubMed and EMBASE databases, each exposure was searched in accompaniment with "AND colon AND (LINE-1 DNA methylation OR DNA methylation OR global methylation)." These varying search terms were used to capture variability in the study of L1 DNA methylation and to include studies that indirectly addressed L1 DNA methylation through "global" methylation measures. Inclusion criteria were as follows: epidemiological studies with primary investigation of at least one of the risk factors of interest to this study and L1 DNA methylation in healthy colon tissue, and publication in English. No year limits were set

on potential studies. References for all studies were also checked for any potential papers matching the inclusion criteria.

2.10.2 Results

With the search criteria specified, 249 papers were found in PubMed, and 372 papers were found in EMBASE. Upon inspection of each paper, only two papers fit the criteria of studying any of the risk of factors of interest in relation to L1 DNA methylation in the healthy human colon. Specifically, these papers reported on the relationship between alcohol consumption, smoking, and BMI on L1 DNA methylation. No additional papers were found through manual reference searches.

Pufulete et al. studied the association between folate status and DNA [^{3}H]methyl incorporation in a UK population of 68 individuals free of colorectal polyps or cancer (an increase in [^{3}H]methyl incorporation reflects a decrease in DNA methylation) (41). The study was able to obtain healthy colon mucosal tissue during routine colonoscopy for analysis of methylation. DNA methylation was analyzed via multivariable linear regression, adjusted for gender, age, BMI, smoking, alcohol consumption, and genotype. Researchers found positive associations approaching statistical significance for relationships between serum folate and serum B_{12} levels and DNA methylation, a negative association approaching significance for smoking, and no association for alcohol consumption (41). An estimate for BMI was not provided. A significant relationship was also observed between *MTHFR* and *MS* genotypes and global DNA methylation. This analysis presents some promising associations between smoking status and global DNA methylation. However, the study had a small sample size, and was potentially

underpowered to detect other associations. Furthermore, only a small set of CRC risk factors was examined in relation to L1 DNA methylation.

Figueiredo et al. analyzed data from an RCT study testing the use of aspirin and folic acid in the prevention of colorectal adenomas in order to study the effects of lifestyle factors and L1 DNA methylation (27). Eligible individuals had to have a history of adenoma, and have removal of at least one adenoma in the past (n=388). Healthy-appearing colon mucosal tissue taken during a surveillance colonoscopy was used for methylation analysis. Independent variables in analysis included: age, sex, BMI, smoking status, alcohol consumption, and dietary folate, B_{12}, and B_6. Researchers did not find statistically significant associations between alcohol, smoking, or BMI and L1 DNA methylation in the analysis. Some limitations of this study included assessment of individuals with a recent history of adenoma, which may have impacted variability in methylation levels in their sample. Furthermore, biopsies from the same individuals were not correlated (r=0.09, r=0.17). Authors were unable to provide an explanation for this lack of correlation, but considering the importance of reliable outcome measurement, this may have negatively impacted their study (27).

2.10.3 Review Summary

This short review summarizes two previously published papers on some of the relationships of interest to this thesis, while highlighting a lack of literature on the associations of interest and L1 DNA methylation in the healthy colon. Specifically, out of 6 proposed risk factors of interest, only 3 have been studied in relation to L1 DNA methylation previously. While these papers inform on associations between alcohol consumption, smoking, and BMI on L1 DNA methylation, there are still significant gaps

in the literature regarding these relationships, as well as the impact of NSAID use, physical activity, and fruit and vegetable consumption on L1 DNA methylation in the healthy colon. Considering this lack of studies, literature addressing L1 methylation in peripheral blood leukocytes was reviewed to develop a background for previous research on the exposures of interest in relation to L1 DNA methylation. For some exposures of interest, only studies analyzing methylation in blood have been conducted previously.

2.11 CRC Risk Factors and LINE-1 DNA Methylation in Peripheral Blood Leukocytes

BMI

Studies analyzing an association between obesity and L1 DNA methylation have had mixed results. A systematic review published in 2015 found 7 epidemiology papers studying the relationship in question in PBLs. Of these studies, 3 found statistically significant associations between BMI and L1 DNA methylation, although associations were not consistently negative (80). Zhang et al. studied the association between specified lifestyle factors and L1 DNA methylation in PBLs in cancer-free subjects enrolled in the Commuting Mode and Inflammatory Response study 2014 (n=165) (81). Overweight or obese subjects had DNA methylation levels significantly lower than those with healthy weight in their analysis. A 2014 analysis of PBLs to study the association between cardiovascular disease risk factors and L1 DNA methylation found a statistically significant positive association between L1 DNA methylation and BMI (β=0.04) (82).

NSAID Use

No studies addressing an association between NSAID use and L1 DNA methylation were found at the time of this review. Although 4 studies have addressed

NSAID use and DNA methylation in site-specific regions of the genome, the biological mechanisms responsible for L1 DNA methylation and site-specific methylation are not necessarily comparable (83). A study analyzing whole genome methylation patterns in relation to NSAID use was conducted in 2015. Researchers did not observe statistically significant relationships between NSAID use and PBL methylation (42).

Physical Activity

Several epidemiologic studies have attempted to quantify the association between physical activity levels and L1 DNA methylation with inconsistent results (44). Out of 8 studies investigating global methylation, 2 found positive associations between physical activity and methylation levels, 5 reported no association, and 1 reported a negative association (43,44). Study design and exposure measurement differed considerably among these studies with cross-sectional, cohort, and randomized controlled trial designs as well as habitual, acute, and intervention exercise regimes used as exposure measures (2,15,81,84-88). These inconsistencies prevent useful comparisons across the literature, and may explain the differences in the directions of effect observed. Furthermore, some interventions may not have been carried out over sufficient time-periods to observe changes in methylation.

Fruit and Vegetable Consumption

As previously discussed, diet represents a complex exposure in relation to L1 DNA methylation. This thesis focuses specifically on fruit and vegetable consumption as a dietary component potentially affecting L1 DNA methylation levels. A previously described study by Zhang and researchers analyzed the relationship between Western and prudent diets and L1 DNA methylation in PBLs. A Western diet was described as having

a high intake of meats, grains, and dairy (85). High fruit and vegetable intake characterize a prudent diet. No associations between diet type and L1 DNA methylation were found using ANOVA methods (85). At the time of this review, no other studies addressing fruit and vegetable intake in relation to L1 DNA methylation were found.

Research addressing the impact of BMI, NSAID use, physical activity, and fruit and vegetable consumption on L1 DNA methylation is lacking. Existing studies addressing these relationships using methylation measures in PBLs have mixed conclusions, and some analyses show a lack of any association between these risk factors and DNA methylation. However, it is important to consider that PBL samples likely do not represent conditions in the healthy colon, and it is inadvisable to conclude lack of association between each CRC risk factor and L1 DNA methylation based on a minor collection of published studies (45).

2.12 Gene-Environment Interactions and DNA Methylation

Gene-environment interactions refer to mechanisms by which certain genotypes and environmental exposures can jointly affect risk of disease or outcome. There are several genes involved in 1-carbon metabolism such as *MTHFR, MTR, MTR, DNMT3B,* and *TS.* Polymorphisms in these genes have been linked to differential methylation patterns in individuals (89). Therefore, it is possible that individuals possessing variant forms of these genes may be more susceptible to aberrant methylation due to lifestyle exposures. Polymorphisms in several of the listed genes have also been linked to CRC risk (90). In addition, these genes have relatively high MAF frequencies (greater than 10%), meaning they have high potential for population impact, in comparison to the study of rare gene variants.

MTHFR codes the enzyme MTHFR, which is responsible for converting dietary folate into 5-methylenetetrahydrofolate, the primary form required for 1-carbon metabolism (90). Individuals that are homozygous for a well-studied polymorphism named *C677T* are referred to as *TT* homozygous, and possess approximately a 70% decrease in MTHFR activity. Approximately 8-20% of North American Caucasians may be *TT* homozygous for the *MTHFR* gene (90).

The *MTR* gene codes MTR, which aids in the production of SAM, a key component of 1-carbon metabolism. An A→G polymorphism at position 2756 has been associated with reduced efficiency and reduced plasma folate levels (90,91). Studies reveal 1-5% of the American population possesses the *GG* genotype (90).

MTRR is responsible for maintaining *MTR* in its active form through production of methionine synthase reductase (90). *GG* homozygotes with an *A66G* polymorphism may have reduced homocysteine levels compared with those with the *AA* allele. Approximately 19-29% of the population possesses the *GG* genotype (90).

DNMT3B is responsible for coding a DNA methyltransferase named DNMT3B which is involved with *de novo* methylation, and is also a part of the folate metabolism pathway (92). The *C46359T* mutation in individuals has been linked to increased promoter region activity of DNMT3B, and increased cancer risk, especially for *TT* variant homozygotes. Epidemiologic studies investigating *DNMT3B* have found North American population frequencies for *TT* to be approximately 20% (93,94).

The *TS* gene creates the thymidylate synthase enzyme, and is involved in 1-carbon metabolism. The enhancer region of this gene contains 28 base-pair tandem repeats. Three repeats (3R), and two repeats (2R) are the most common number of

repeats of the 28 base-pair region. In vitro, the 3R/3R genotype has been associated with reduced plasma folate levels. It is hypothesized that the 2R polymorphism may have a protective effect on cancer risk. In the American population, 19-23% of the Caucasian population are homozygous for 2R/2R (90).

In addition, *DHFR, RFCI, PEMT, MTHFD, BHMT, SHMT, TYMS*, and *CHDH* genes are also implicated in proper functioning of one-carbon metabolism (95,96). However, their roles in one-carbon metabolism are not uniformly established in the literature, with limited information on their associated cancer risk, and propensity to affect L1 DNA methylation (97-100).

Despite the described relationships between each gene and one-carbon metabolism, there is lack of functional understanding of the effect of specific polymorphisms on L1 DNA methylation. Aside from *MTHFR*, relationships between the remaining genes and L1 DNA methylation are not uniformly described in the literature. A recent review of these genes highlights the lack of information available on the exact mechanisms and effects of even the more commonly studied *MTR* and *MTRR* genes on both metabolic processes and cancer risk (90). Difficulty in the study and interpretation of the effects of specific polymorphisms stems from the potential that any observed differences between genotypes may be due to the presence of another linked polymorphism with opposing effects that is yet to be discovered, and literature on the interactions between these polymorphisms and risk factors on CRC risk is limited (90). Significant gene-environment interactions have been reported for *MTHFR* with alcohol consumption, and dietary folate intake on the relationship with CRC, with patterns of increased risk among those with gene variants (90). One previous study also found a

significant interaction for *MTR* and alcohol consumption on the relationship with CRC, where individuals with the gene variant of interest had reduced methylation (101). No significant interactions by *MTRR* and lifestyle exposures have been reported in the literature (90,102).

2.13 Study Rationale

Colorectal cancer is the 2nd most common cancer diagnosis in Canada, accounting for approximately 13% of all diagnoses. Screening programs which detect precancerous adenomatous polyps are in place across Canada and have slightly reduced CRC incidence since 2000 (103). However, continuing research that may help identify causes of CRC is integral to future progress.

Epigenetic modification of the human genome and its role in cancer development has stimulated substantial research in recent years (30,31,104). However, the majority of these papers do not address epigenetic changes occurring in the healthy colon. Rather, most studies analyze tissue samples taken from blood, or adenoma/CRC tissue (37,52,53,56,81,85,105). Studies examining DNA methylation in blood are not ideal as blood is not the target tissue of interest, and methylation measures in blood only moderately correlate with measures in colon tissue (45). Studies focusing on adenoma or CRC tissue are also unsuitable for thesis objectives as aberrant methylation in this tissue may be a consequence rather than a cause of disease.

The proposed investigation is based within a study that examined L1 DNA methylation in healthy colon tissue in relation to risk of adenoma. This study builds on previous research relating lifestyle risk factors to DNA methylation and CRC by informing on potential mechanisms through which known risk factors may influence

methylation levels, ultimately leading to CRC. Changes in methylation may be influenced by lifestyle risk factors for CRC or from gene-environment interactions in which exposures may have a stronger impact on those individuals that possess variant alleles for genes involved in methylation (89).

Finally, this research assesses associations in a more comprehensive set of CRC risk factors than previously done. Considering the difficulty of following up exposures to outcomes in cancer research due to long latency and rare outcomes, focusing on intermediate factors on the pathway to CRC will facilitate improved understanding of early risk factors that may lead to CRC.

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Chapter 3

Study Design, Methods, & Preliminary Analyses

3.1 Introduction

This chapter provides an overview of the specific study objectives for the manuscript chapters to follow. Considering that thesis research was a nested design within a previously completed cross-sectional investigation, this section serves to provide additional details regarding the original study design and methods. In addition, due to manuscript content constraints, preliminary analysis conducted prior to analyses for the two manuscripts is also included in this chapter.

3.2 Study Objectives and Hypotheses

1.) To examine the relationship between recognized colorectal cancer (CRC) risk factors including alcohol consumption, smoking, body mass index (BMI), fruit and vegetable consumption, physical activity, and non-steroidal anti-inflammatory (NSAID) use, and Long interspersed nuclear element-1 (L1) DNA methylation in normal-appearing colon mucosal tissue.

Hypothesis: Lower levels of L1 DNA methylation are expected in individuals exposed to CRC risk factors including alcohol consumption, smoking and high BMI, and increased levels of L1 DNA methylation are expected in individuals regularly consuming NSAIDs, engaging in physical activity, and consuming fruits and vegetables.

2.) To examine the relationship between polymorphisms in genes linked to DNA methylation, including *MTHFR, MTR,* and *MTRR,* and test for effect modification between the set of CRC risk factors and gene polymorphisms (1,2).

Hypothesis: Genes linked to one-carbon metabolism will be associated with L1 DNA methylation, and stronger associations between CRC risk factors and aberrant DNA methylation are expected in individuals already susceptible due to genotypic differences in proposed genes.

3.3 Original Study

This study is nested in a cross-sectional analysis of L1 DNA methylation and colorectal adenoma risk in patients aged 40-65 in Kingston, Ontario (3). Institutional ethics approval was obtained from the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (File No. 6004521) and all subjects provided informed consent for the original investigation. We acknowledge the support from the Canadian Cancer Society as well as the researchers involved in the original study conceptualization, and data collection: Janet E Ashbury, Sherryl A Taylor, M Yat Tse, Stephen C Pang, Jacob A Louw, and Stephen J Vanner (3). This nested analyses received a Letter of Approval from Queen's Health Sciences Research Ethics Board (Appendix A).

Patient recruitment for the study occurred from 2009-2012. Patients scheduled to undergo a screening colonoscopy at Hotel Dieu Hospital were recruited 1-4 months prior to their scheduled appointment by mail. 728 subjects met the initial eligibility criteria, of which 444 consented to participation (response rate=66%). After exclusion criteria were applied, a total of 317 individuals were enrolled in the study. Two pinch biopsies of normal-appearing colon mucosal tissues were collected during the procedure, along with a fasting blood sample. 209 participants had a normal colonoscopy, and 108 had at least one confirmed adenoma. The main objective of the original investigation was to better

understand the role of L1 DNA methylation in the early stages of adenoma development at the population level. By collecting and analyzing normal-appearing colon mucosal tissue, the researchers hoped to investigate changes occurring in the colon prior to cancer.

3.3.1 Study Participants

The study population started as a cohort of colonoscopy patients, however, several exclusion criteria were required to define a homogenous adenoma outcome. Patients with serrated adenomas, sessile serrated adenomas, or only hyperplastic polyps were excluded as these growths are separately defined in the literature, and likely occur by distinct biological mechanisms than those of interest to the study (3,4). Individuals with diagnosed inflammatory bowel disease and those with known genetic disorders linked to CRC were also excluded from this study due to increased risk of adenoma by mechanisms separate from those under investigation (3,4). Finally, individuals previously diagnosed with any form of cancer (except non-melanoma skin cancer) were also excluded due to the same concerns as defined above. Participants completed a questionnaire to ascertain information on risk factors associated with CRC for potential exclusion from the study population.

3.3.2 Data Collection

Information on L1 DNA methylation in the 317 participants was collected through laboratory analysis at Queen's University. Laboratory methods in DNA processing and isolation are described in detail in a later passage (3). Briefly, DNA from colon mucosal samples was collected using the 5-PRIME DNA isolation kit. DNA was then bisulfite-converted in order to distinguish unmethylated cytosines from 5-methylcytosine. High-resolution melt curve (HRMC) analysis was then conducted on 8

CpG sites of interest in the L1 consensus promoter region (5). HRMCs allow for quantification of L1 DNA methylation in the genome using polymerase chain reactions (PCR), and cover a full range of methylation levels, including the biologically relevant range in human specimens of 50-90% (5). The two pinch biopsies collected from each individual were averaged to obtain final L1 methylation measures. Correlation between each pair of biopsies was 0.66, and deemed acceptable in the measurement of a field effect (3).

Measurements of exposures of interest come from self-reported survey questions. Most survey questions sought exposure information up to at least 1 year prior to colonoscopy. Exposure information during this time-window is important as DNA methylation patterns are regarded as relatively stable, with changes due to lifestyle exposures occurring over longer time-periods (6-12 months) (6-9).

Information on reliability or reproducibility is not available for the questionnaire in its entirety, as it was designed specifically for the original cross-sectional study. However, physical activity and diet sections of the questionnaire were adapted from previously published surveys. Specifically, the International Physical Activity-Short Form (IPAQ-SF) was used to assess physical activity. The questionnaire has acceptable repeatability, with 75% of correlation coefficients reporting a value of 0.65 or higher in a recent review (10). Criterion validity comparing IPAQ-SF results to accelerometer values reveal fair to moderate agreement (nonparametric Spearman's correlation coefficient=0.30) (10). The dietary questionnaire was guided by a paper on the Abbreviated Dietary Interview, which states that a large questionnaire comprising of 128 foods had high agreement with a shorter, select list of 20 foods (Kappa correlation

coefficients from 0.61-0.88) (11). Detailed information regarding exposure ascertainment and conceptualization of each variable is provided further below.

3.3.3 Genotyping

Measurement of genetic polymorphisms of interest in *MTHFR, MTR,* and *MTRR* genes was conducted at Genome Quebec, in Montreal, Canada using the MassARRAY iPLEX Gold–SNP Genotyping assay. Study analyses sought to determine associations between normal and variant genotypes for the listed genes of interest and CRC risk factors. In order to maximize study power to determine differences in between the genotypes, heterozygous individuals were grouped with homozygous variant individuals. Previous analyses of genes selected for this study have also collapsed heterozygote and homozygote variant genotypes for analysis (12). Information on the genes of interest and expected genotype frequencies are presented in Table 3-1. Briefly, all genes were in HWE except for *MTHFR* (p-value=0.04). However, this was not expected to affect analyses, and there was potential for a lack of HWE in the genes studied considering the population under study was a non-representative screening population. All genes had distributions that were similar to those reported by NCBI based on large genomic databases (13).

3.4 Measuring LINE-1 DNA Methylation

L1 DNA methylation is a specific type of epigenetic modification that occurs on L1 elements across the genome, and is distinct from gene-specific methylation. These elements are typically methylated under normal conditions to retain genomic stability (17,18).

Table 3-1. Gene information and Hardy-Weinberg Equilibrium (HWE) analysis

Gene SNP	N (%)	Percent Expected	HWE X^2 Results	Gene Function[a]	Population Frequency and MAF[b]
MTHFR-rs1801133	*CC*=153 (48.26)	45.78	0.04	Codes for enzyme responsible for converting dietary folate for 1-carbon metabolism (1CM).	Reported frequency for *TT* in white populations is 8-20% (12).
	CT=123 (38.80)	43.75			
	TT=41 (12.93)	10.24		*TT* genotype associated with higher risk of colorectal carcinoma, adenoma, and hyperplastic polyps.(12)	RefSNP MAFs: 24-30% (13).
MTR-rs1805087	*AA*=199 (62.78)	63.94	0.19	Codes for enzyme aiding in production of SAM (a key component of 1CM). *GG* is linked to reduced functioning.	Reported frequency for *GG* in white populations is 2-3% (12).
	AG=109 (34.38)	32.04			
	GG=9 (2.84)	4.01			
				GG genotype associated with increased risk of CRC.(14)	RefSNP MAF: 20-22% (13).
MTRR-rs1801394	*AA*=61 (19.14)	20.20	0.49	Responsible for maintaining *MTR* in its active form. *GG* linked to reduced function.	Reported frequency 28-29% for two case-control studies for *GG* in white populations (15,16).
	AG=163 (51.42)	49.49			
	GG=93 (29.34)	30.30		*GG* associated with increased risk of CRC.(12)	
					RefSNP MAF: 36-47%(13).

[a]Literature on the associations between each genotype and CRC adenoma risk shows overall directions of effect suggesting increased risk, however this literature is sparse for *MTR* and *MTRR*. Furthermore, several of the studies do not have statistically significant risk estimates.
[b]RefSNP NCBI resources for MAFs include 1000 Genomes, ExAC, GO-ESP, and TOPMED genome projects (13).

About 80% of CpG sites are found in repetitive sequences such as L1 elements. L1 elements comprise about 20% of the genome (19). Therefore, a measure of L1 DNA

methylation across the genome must be able to provide information on methylation across the approximately 500,000 copies of L1 elements in the genome. For the PCR reaction required for measurement of methylation in this study, primers designed for the L1 consensus promoter region encompassing 8 specifically selected CpG sites were used to ascertain an average measure of L1 methylation across the genome. In application, PCR allows for the reading and sequencing of thousands of these sites in the same promoter consensus sequence region of L1 elements. It is this process that allows these 8 sites to represent methylation across L1 elements throughout the DNA molecule. Once DNA within the primers was amplified, high-resolution melt curves were generated for each sample, and compared to melt-curves with known levels of unmethylated and methylated cytosines.

The original protocol paper for this method of DNA methylation quantification reports low intra- and inter-assay coefficients of variation, at 1.44% and 0.49% respectively (5). That is, there was little variation in replicate samples on the same PCR plate, as well as replicate samples on separate PCR plates, respectively. Within the samples obtained for this thesis, the intra-assay coefficient of variation within tissue replicates was 1.84%, and inter-assay coefficient of variation (within PCR plates) was 0.89%. Taken together, these values indicate a method of measuring L1 DNA methylation across the genome that is reproducible and reliable. Correlations were also computed for the majority of samples analyzed, with a Pearson's correlation value of 0.66. Final methylation values represent average methylation across all CpG sites, both tissue biopsies, and across all cell types in each biopsy for each patient. Due to financial and time constraints, all biopsied samples could not be run separately, and methylation

values were based on one combined DNA extraction sample for 55 subjects.

3.5 Statistical Analyses

L1 DNA methylation was the outcome of interest. It was measured on a continuous scale, approximately normally distributed, and was standardized (mean=0, standard deviation=1)-as done in previous publications involving the data (3,20).

Objective 1 was addressed by conducting multivariable linear regression (MLR) analysis on the outcome of interest in order to determine if known risk factors for CRC are associated with L1 DNA methylation. Age and sex were forced into the models as established predictors of L1 DNA methylation, while ethnicity, and serum levels of folate and B_{12} were evaluated as potential confounders using a backwards elimination approach (liberal p-value of 0.15) (6, 21). All modeling was done in SAS 9.4.

Objective 2 evaluated the interaction between polymorphisms and established CRC risk factors on L1 DNA methylation using MLR. The relationships between CRC risk factors and L1 DNA methylation were modeled separately for each gene. Interactions were evaluated at a predetermined alpha of 0.05. Age and sex were forced into the models as established predictors of L1 DNA methylation, while ethnicity, and serum levels of folate and B_{12} were evaluated as potential confounders using a backwards elimination approach (liberal p-value of 0.15) (6,21). Stratified coefficients and p-values by genotype for each variable of interest were extracted from the same model using contrast terms in SAS 9.4.

3.6 *A-priori* Power Calculations

A-priori power calculations were conducted for all exposures of interest in this research. Due to the unavailability of data at the time of calculation, exposure

distributions could not be directly obtained for *a-priori* power calculation. Exposure prevalence for each variable was estimated from published frequencies on the reported data (3,20). NSAID use had not yet been investigated in previous analysis, and distribution information was not readily available for an Ontario population 40-65 years of age. Prevalence of prescription NSAID use was therefore estimated from population level analysis in an Ontario population aged 66+ from the year 2002 as a surrogate prevalence measure in the data (22).

For Objective 1, minimal detectable effects were estimated for each exposure of interest using a fixed sample size of 317, 80% power, and statistical significance of p=0.05. L1 DNA methylation was considered to be a normally distributed outcome with a mean of 0, and a standard deviation of 1. Detectable effect calculations were based on a two-sample t-test, comparing mean levels of L1 methylation in two categories of the independent variable (23). Therefore, reported effect sizes represented the minimum detectable effect per standard deviation, based on exposure prevalence. Detectable effect sizes ranged from 0.316 of a standard deviation for some genetic polymorphisms to 0.474 of a standard deviation for smoking (Table 3-2). This study had limited statistical power to examine interactions between the set of risk factors and genotypes.

3.6.1 Missing Data

The project dataset contained colon tissue methylation values for 317 patients, however, information on exposures of interest was incomplete for some participants. If a complete-case analysis method was used for data analysis, only 290 observations would have been available for study, limiting the statistical power to detect relationships of interest. Therefore, it was important to fill in values for missing observations.

Table 3-2. *A-priori* power calculations for all independent variables

Exposure	Expected N	Minimal detectable effect size per SD*
Alcohol Use (20)	Abstainers=54 Low=183 Moderate=105	Between Abstainers and Moderate=0.472.
Smoking (20)	Never=183 Past=115 Current=44	Between Never and Current=0.474.
BMI (24)	<18.5kg/m^2=6 18.5kg/m^2-24.99=143 25-29.99kg/m^2=114 ≥30kg/m^2=54	Between 18.5kg/m^2-24.99 and ≥30kg/m^2=0.45
Fruit and Vegetable Intake	Q1=79 Q2=79 Q3=79 Q4=80	Between Q1-Q4=0.449
NSAID Use (22)	Yes=127 No=190	0.322
MTHFR polymorphism	CC=187 CT/TT=136.5	0.318
MTR polymorphism	AA=194 AG/GG=123	0.32
MTRR polymorphism	AA=128 AG/GG=189	0.323
Sex (3)	Male=137 Female=180	0.318
Age (3)	40-49=55 50-54=98 55-69=86 60-65=78	0.498

*Detectable effect for mean difference of the standardized outcome variable. Represents the detectable effect per standard deviation with 80% power and significance of 0.05.

Original study questionnaires were obtained for patients with missing values, and the pertaining information was added to the final dataset. After this process, a total of 303 complete cases were available. For truly missing values, stochastic regression imputation was used to address the remaining 14 values. It is worthwhile to note that the dataset was mostly complete before imputation, and although imputation was used to fill in missing values for 14 different observations, 5 of these values were for potential confounders, and not main exposures of interest.

Stochastic regression imputation was chosen because it does not decrease variability in the data that is seen in mean imputation or regression imputation (25,26). A multiple imputation approach was considered problematic for some proposed analyses that may have required bootstrapping, and therefore a single imputation was used to fill in missing observations.

Due to the limited number of exposures for which values were imputed, this process was expected to have a negligible effect on further analysis, and is not further discussed in this thesis.

3.7 Analyses Prior to Manuscript Preparation and Conceptualization of Exposures

As previously described, demographic, lifestyle exposure, and confounder information was obtained from a self-administered questionnaire completed prior to the colonoscopy appointment. Each exposure and confounder was carefully considered in various representations before moving forward with regression analysis.

Participants were asked about their alcohol consumption as a weekly average, representative of consumption over the past year for beer, wine, liquor, and coolers. Average consumption over the past week of each beverage was used to calculate an

average for grams of alcohol consumed per day. Cut-points for data categorization were based on sex-specific guidelines outlined by 2017 published Canadian Guidelines on Substance Abuse and Addiction (27). A drink was defined as 13.6g of alcohol. In both men and women, abstainers were those whose alcohol consumption was reported as 0g. In men, "light" consumers were those who consumed no more than 2 drinks per day on average, "moderate" consumers were those consuming 3-4 drinks per day, and "heavy" consumers were those consuming 5 or more drinks per day. In women, "light" consumers were those having 1 or fewer drinks per day, "moderate" consumers were those having 2-3 drinks per day, and "heavy" consumers were those having 4 or more drinks per day, on average. Due to the lack of heavy consumers in the dataset, moderate and heavy consumption categories were combined.

Smoking status was categorized into 'never smoker', 'previous smoker', and 'current smoker'. A participant was considered a current smoker if they had smoked at least one cigarette per day in the past year, and as a previous smoker if they had smoked at least one cigarette a day for six or more months during any point in their lives.

BMI was obtained from height and weight measurements recorded in patient reports prior to colonoscopy. Categories of BMI were created based on established cut-points (28). Only one individual in the dataset was underweight, therefore, this category was merged with normal weight individuals. Individuals with a BMI below 25 were categorized into the normal weight category, individuals with a BMI between 25 and 30 were considered overweight, and individuals with a BMI at or above 30 were considered obese (28).

NSAID use is a novel exposure of interest in relation to L1 DNA methylation, making this a particularly important research question within this thesis. However, the lack of previous research on this subject was problematic for selecting the most appropriate representation. Epidemiologic studies of NSAID use are heterogeneous in their exposure assessment, as some studies focus on the length of time of use (captured in months or years), the quantity of daily dose, the type of NSAID consumed, or any combination of the three (29-31). Overall, there does not appear to be an accepted standard for reporting on NSAID use.

As the original study questionnaire was not created with the proposed analysis in mind, NSAID use was measured as months of use in the past year for both prescription and non-prescription NSAIDs, as well as months of use in the past 10 years for both prescription and non-prescription NSAIDs. For this thesis, a focus on the last year of use was selected, as this corresponded best with exposure windows for the other risk factors of interest. A categorical variable was created to reflect NSAID use in which number of months of continuous use in the past year of either prescription or non-prescription NSAIDs was used to categorize use into “no use”, “less than one year of use” (short-term use), or “full year of use” (long-term use). Although this measure is not able to account for differences in prescription and non-prescription use, or among daily-dose or type of NSAID, given the availability of exposure information, this approach is likely the best way to obtain a variable representative of the NSAID dose for each individual. Furthermore, different variable classification by prescription and non-prescription NSAIDs did not point to statistically significant differences in initial analysis (data not shown).

Physical activity was measured with an adapted version of the IPAQ-SF (10). This questionnaire is a widely used questionnaire for assessment of physical activity, and assesses an individual's average physical activity levels week-to-week for the past year (32). Activity is divided into vigorous and moderate, and reported as the number of days per week, and hours per day in both categories. Metabolic Equivalent of Task (MET) minutes were calculated based on reported values, and approximately equal quartiles of physical activity were created for analyses (33).

Dietary information collected in the self-administered questionnaire was adapted from the Abbreviated Dietary Interview (11). Specifically, participants were asked about consumption of 42 common fruits and vegetables. This information was collected as the frequency of consumption per week, for the past year. The frequency of consumption was summed, and approximately equal quartiles were created for analysis.

Age and sex are strong predictors of L1 DNA methylation, and were forced into all regression models (6,21). Potential confounders investigated including ethnicity, serum-folate, and serum-B_{12} levels. Age and sex were determined through patient reports, and ethnicity was categorized as "Caucasian" or "Other" due to a small proportion of non-Caucasians in the study population (7%). Serum-folate and B_{12} levels were collected from lab results at Kingston General Hospital. These selected confounders of interest were established predictors of L1 DNA methylation (34-37).

L1 DNA methylation values were standardized for analyses. Bivariate relationships of exposures and confounders with L1 DNA methylation were assessed using linear regression, while controlling for age and sex - two important determinants of L1 DNA methylation (6,21). Where possible, continuous versions of variables and

confounders were assessed as predictors of L1 DNA methylation using quadratic terms, in parallel with variable categorization (Appendix B, Table B-1). Relationships between continuous versions of each variable, as well as with quadratic versions of each variable with L1 DNA methylation were not significant.

Descriptive statistics for each continuous exposure are provided in Appendix B, Table B-1. No linear relationships were significant in the described preliminary analysis. Data from this initial analysis did not point to the presence of strong relationships between each exposure and outcome, and no exposures were significantly related to L1 DNA methylation in bivariate analysis. Relationships between the covariates were also assessed with appropriate statistical tests (Appendix B, Table B-2). This also allowed for better understanding of the relationships between all variables of interest.

3.8 Assumptions of Linear Regression

Before beginning planned analysis, it was vital to test the assumptions of linear regression. The four assumptions of linear regression include: *normality, independence, homoscedasticity of residuals,* and *linearity* (38). In the context of this study, it is important to consider normality of the outcome variable, and homoscedasticity of the error terms. However, independence of the observations was assumed within this nested analysis, and testing for a linear relationship between the independent and dependent variable is a part of the specific thesis objectives. Normality in the error distribution of L1 DNA methylation was assessed using a quantile-quantile plot. Plotting normal quantiles with those of the error distribution did not suggest a deviation from normality. Homoscedasticity of the error terms was tested using by plotting L1 DNA methylation

with the studentized Pearson residual for each observation. Plotting results did not point to a concerning lack of homoscedasticity.

Finally, Cook's Distance was used to check for observations that may unduly influence the analysis. Cook's Distance measures the effect of deleting an observation from analysis. A large Cook's Distance merits further examination. Although several cutoffs are used in the literature, a value greater than one is considered a reasonable cutoff (39,40). No values had a Cook's Distance greater than 0.06, and therefore, no single observation was considered an outlier that may distort analysis results.

3.9 Conclusion

This chapter detailed the specific methodological considerations required before analyses presented in the following chapters could be completed. Specifically, variable conceptualization and genotype frequencies needed to be assessed before Objectives 1 and 2 could be completed. The following chapters present two manuscripts that describe the results and conclusions of Objectives 1 and 2, and will be submitted to *Cancer Causes and Control* (Brief Communication) and *Epigenetics*. The chapters have been specifically formatted to the respective journal guidelines. Objectives 1 and 2 were conceptualized as distinct analyses during the thesis proposal, and considering the extensive work completed, it was deemed necessary to separate objectives into individual manuscripts.

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Chapter 4

The relationship between colorectal cancer risk factors and LINE-1 DNA methylation in healthy colon tissue

4.1 Abstract

Purpose: LINE-1 DNA methylation is an important, potentially modifiable epigenetic process. While aberrant methylation in blood is linked to CRC risk, studies of methylation in the tissue of interest are limited. This research examines associations between established colorectal cancer risk factors and LINE-1 DNA methylation in healthy colon tissue to investigate LINE-1 DNA methylation as a mechanism by which established risk factors may lead to carcinogenesis.

Materials and Methods: The study population consisted of 317 individuals undergoing colonoscopy screening. Multivariable linear regression was employed to examine associations of alcohol consumption, smoking, BMI, NSAID use, physical activity, and fruit and vegetable consumption with LINE-1 DNA methylation, while controlling for confounders.

Results: Alcohol consumption, smoking, BMI, NSAID use, physical activity, and fruit and vegetable consumption were not related to LINE-1 DNA methylation in this population.

Conclusion: This work presents novel investigations of relationships between NSAID use, and fruit and vegetable consumption with LINE-1 DNA methylation in the tissue of interest, and is an important addition to the sparse literature in this field. DNA methylation is potentially modifiable, and improved understanding of factors impacting

methylation are important in developing targeted interventions aimed at stabilizing methylation in individuals in which aberrant methylation is identified.

4.2 Introduction

DNA methylation is a key epigenetic process controlling transcription of the DNA molecule. It involves the transfer of a methyl-group to cytosine residues in CpG dinucleotide pairs, the majority of which are found in repetitive sequences (80%) (1). Repetitive sequences are multiple copies of DNA that are normally methylated to maintain genomic stability. L1 (long interspersed nuclear element-1) is the most widely studied category of repetitive elements, and comprises approximately 17% of the human genome (1). L1 DNA methylation is modifiable over longer time periods, but is shown to be stable in studies measuring variation week-to-week, and month-to-month (2,3). Hypomethylation of L1 repeats is associated with genomic instability and is linked with increased risk of colorectal cancer, as well as other cancer sites, including head and neck, stomach, ovarian, cervical, lung, and liver cancers (4-8). These established relationships support the study of L1 DNA methylation as an intermediate endpoint to carcinogenesis in the colon.

Studying DNA methylation as a potential mechanism of action in the relationship between CRC risk factors and cancer is particularly important, as it is a modifiable process (2,3). An understanding of factors impacting methylation is crucial to the development of targeted interventions based on lifestyle changes, or dietary supplementation aimed at stabilizing L1 DNA methylation in individuals in which aberrant methylation is identified. These targeted interventions will require several years

of research and testing, however, they highlight the potential and importance of studying modifiable biomarkers in the present day.

DNA methylation patterns are tissue-specific (9). Differences in methylation stability, cell type mixture, and efficiency of critical metabolic processes across different tissue types warrant examination of methylation at different sites (9), (10-12). Peripheral blood leukocytes (PBLs) are a popular tissue of choice for the analysis of DNA methylation, as they are a convenient source of DNA. However, methylation patterns found in PBL samples may not reflect methylation occurring in tissues of interest, such as colon mucosal tissue. Analysis of L1 DNA methylation in PBLs and colon mucosal tissue samples reveal weak correlations in methylation patterns between the two tissues (r=0.36) (13). Although comparison of L1 levels in PBL and colon tissue is limited, similar analyses focusing on PBL and kidney tissues also report low correlation values (r=0.08) (14). These findings suggest caution when comparing results from methylation analysis of PBLs, and supports the importance of measurement of methylation directly from tissues of interest.

Furthermore, recent research suggests that variability in DNA methylation found in PBLs may be a function of the type of blood cells captured in analysis, rather than person-to-person variability due to lifestyle factors, and although this concern may remain in regard to cell type distributions in tissues of interest, it may be mitigated depending on the type of tissue, and sampling procedure (9).

Colorectal cancer is a leading cause of cancer-related deaths in North America (15). Colorectal tumour development follows an established sequence from aberrant proliferative epithelial dysplasia, to colorectal adenomatous polyps (adenoma), to CRC

(15). Adenomas are a recognized precursor in the pathogenesis of CRC (15). Although all adenomas do not result in CRC, most cases of CRC arise from adenomas in the colon, and approximately 50% of the general population will develop an adenoma in their lifetime (15).

The relationships between alcohol consumption, smoking, body mass index (BMI), physical activity, non-steroidal anti-inflammatory drug (NSAID) use, and fruit and vegetable consumption with CRC are established (1,4,5,7). Previous research on biologically plausible mechanisms, including one-carbon metabolism and inflammation, relate the listed exposures of interest to L1 DNA methylation, and further supports study of these relationships as intermediate processes on the pathway to carcinogenesis (4-8).

The objective of this study was to examine the relationship between established CRC risk factors with L1 DNA methylation in the healthy colon. We hypothesized that alcohol consumption, smoking, and high BMI would be associated with reduced methylation, while NSAID use, physical activity, and fruit and vegetable consumption would be associated with methylation maintenance.

4.3 Materials and Methods

This analysis was nested within a cross-sectional study investigating L1 DNA methylation and risk of colorectal adenoma, a precursor to CRC (16). Healthy patients aged 40-65 years old scheduled for a routine colonoscopy at Hotel Dieu Hospital (Kingston, Canada) were recruited between 2009-2012.

DNA for methylation analysis was isolated from healthy colon tissue samples. High-resolution melt curve analysis was used to quantify L1 loci-specific methylation values. A complete description of the study population and laboratory methods is detailed

elsewhere (16). Briefly, DNA was isolated from leukocytes and purified using the 5-PRIME DNA isolation kit (Inter Medico, Markham, ON, Canada). DNA concentrations were determined using Quant-iT™ PicoGreen®, before storage at −20°C. DNA was bisulfite converted, and PCR was carried out using primers designed for the L1 consensus promoter region, which targeted 8 CpG sites representative of L1 DNA methylation across the genome. High-resolution melt curve (HRMC) analysis, a real-time fluorescence-based polymerase chain reaction, was used to quantify L1 loci-specific methylation values (16).

Demographic and lifestyle exposure information was obtained from a self-administered questionnaire completed prior to colonoscopy. Alcohol consumption cut-points were based on sex-specific guidelines outlined by 2017 published Canadian Guidelines on Substance Abuse and Addiction (17). Due to the lack of heavy consumers in the dataset (N=1), moderate and heavy consumption categories were combined.

Smoking status was defined as ‘never smoker’, ‘previous smoker’, and ‘current smoker’. A participant was classified as a current smoker if they had smoked at least one cigarette a day for the past year, and as a previous smoker if they had smoked at least one cigarette a day for six months or more at any point during their life.

Categories of BMI were based on established cut-points. Only one individual in the dataset was underweight, therefore, this category was merged with normal weight individuals. Individuals with a BMI below 25 were classified as having a normal BMI, individuals with a BMI between 25 and 30 were classified as overweight, and individuals with a BMI at or above 30 were classified as obese.

Epidemiologic studies of NSAID use are heterogeneous in their exposure assessment, as some studies focus on the length of time of use (captured in months or years), the quantity of daily dose, the type of NSAID consumed, or any combination of the three (5,18). Overall, there does not appear to be an accepted standard for reporting on NSAID use. For this analysis, number of months of continuous use in the past year of prescription or non-prescription NSAIDs was used to categorize use into "no use", "less than one year of use" (short-term), or "1 full year of use" (long-term) for analysis.

Physical activity was measured with a questionnaire adapted from the short-form International Physical Activity Questionnaire. Approximately equal quartiles of physical activity, represented as Metabolic Equivalent of Task minutes per week were used in our analyses.

Fruit and vegetable consumption information was collected from a modified version of the Abbreviated Dietary Interview. Participants were asked about consumption of 42 common fruits and vegetables. This information was collected as the frequency of consumption per week, for the past year.

Established determinants of L1 DNA methylation were considered confounders in analysis and included age, sex, ethnicity, serum-folate, and serum-B_{12} levels (7,19).

Multivariable linear regression was used to assess the relationship between CRC risk factors and L1 DNA methylation in healthy colon tissue. Age and sex were forced into all modeling, and a backwards elimination procedure ($p=0.15$) was used to evaluate ethnicity, serum folate, and serum B_{12} levels as potentially confounding variables. Trend tests were also conducted for each categorical exposure of interest by assigning integer values to each category, and then assessing the variable as continuous.

All statistical tests were conducted in SAS 9.4.

4.4 Results

L1 DNA methylation was normally distributed (μ=88.17, σ=3.62). Characteristics of the study population are described in Table 1. Briefly, 57% of participants were female, 93% were Caucasian, and 59% were between the ages of 50-60. About one third of the study population had an adenoma at the time of colonoscopy (34%). Females had lower methylation values than males (p=0.001), while age was not significantly related to L1 DNA methylation (p=0.15).

The relationship between each exposure of interest and L1 DNA methylation is presented in Table 2. No potential confounders were selected during backwards elimination, and therefore only sex and age were included as confounders in regression analyses. No CRC risk factors were significantly related to L1 DNA methylation after confounder adjustment, and after mutual adjustment for all other exposures. There were no clear dose-response relationships between each exposure and the outcome, as measures of effect differed in directionality across different categories of each exposure.

4.5 Discussion

The role of decreased methylation in carcinogenic processes is well established, however, there is a lack of previous literature assessing the potential for lifestyle exposures to affect methylation levels in the healthy colon (1). In previous research, PBLs have been a convenient and popular tissue of choice for the analysis of DNA methylation. However, DNA methylation patterns are tissue-specific (16), and variability in L1 DNA methylation found in PBLs may be a function of the type of blood cells

Table 1. The association of study population characteristics with LINE-1 DNA methylation in colon tissue (N=317)

Characteristic	N (%)	Coefficient[a]	p-value
Sex			0.001
Male	137 (43.22)	Ref.	
Female	180 (56.78)	-0.37	0.001
Age			0.15
39-50	55 (17.10)	Ref.	
50-60	184 (58.39)	-0.03	0.86
60+	78 (24.52)	0.23	0.19
Adenoma			0.19
No	209 (65.93)	Ref.	
Yes	108 (34.07)	-0.16	0.18
Ethnicity			0.78
Caucasian	295 (93.44)	Ref.	
Other	22 (6.56)	0.06	0.78

[a]Coefficients in relation to standardized LINE-1 DNA methylation, adjusted for age and sex

captured in analysis (9). These limitations in surrogate tissue analysis motivated current analysis of L1 DNA methylation directly in healthy colon tissue.

One-carbon metabolism and inflammation represent two mechanisms of action that may relate CRC risk factors to L1 DNA methylation occurring in the healthy colon and further supported current analysis. One-carbon metabolism comprises of a group of related pathways at the cellular level that are involved in amino acid and nucleotide metabolism (4). Methyltransferases facilitate these pathways, and use S-adenosylmethionine (SAM) as a methyl donor group. Methyltransferases require tetrahydrofolate (THF), a form of folate, for the reaction. Once SAM is activated to form methionine, the chemical is used for various cellular reactions, including DNA methylation (4). Other dietary vitamins such as B_{12}, B_6, and B_2 are also related to one-

Table 2. Multivariable linear regression analysis between each exposure of interest and LINE-1 DNA methylation (N=317)

Lifestyle Exposure	N (%)	Coefficient[a]	p-value	Coefficient[b]	p-value
Alcohol Consumption[c]			0.13		0.14
Abstainers	46 (14.51)	Ref.		Ref.	
Light	202 (63.72)	-0.19	0.23	-0.18	0.25
Moderate	69 (21.77)	0.07	0.69	0.08	0.67
Trend			0.71		0.73
Smoking			0.09		0.10
Never	170 (53.64)	Ref.		Ref.	
Former	106 (33.44)	-0.16	0.19	-0.14	0.24
Current	41 (53.63)	0.24	0.15	0.25	0.13
Trend			0.30		0.30
BMI			0.40		0.32
Underweight/Normal	107 (29.65)	Ref.		Ref.	
Overweight	116 (36.59)	-0.02	0.85	-0.03	0.81
Obese	94 (33.75)	0.16	0.26	0.17	0.23
Trend			0.31		0.31
NSAID Use[d]			0.15		0.20
No use	206 (64.98)	Ref.		Ref.	
Less than one year of use (short-term)	47 (14.83)	-0.19	0.24	-0.19	0.23
Full year of use (long-term)	64 (20.19)	0.19	0.18	0.16	0.24
Trend			0.36		0.35
Physical Activity (MET minutes per week)			0.11		0.16

<1908	83 (26.18)	Ref.		Ref.	
1908-3745	78 (24.61)	-0.007	0.96	-0.01	0.93
3745-6542	79 (24.92)	-0.14	0.34	-0.11	0.45
>6542	77 (24.29)	0.24	0.11	0.24	0.12
Trend			0.13		0.13
Fruit and Vegetable Consumption (number of servings per day)			0.23		0.18
<2.9	79 (24.92)	Ref.		Ref.	
2.9-4.1	79 (24.92)	0.13	0.41	0.12	0.45
4.1-5.72	77 (24.29)	-0.18	0.25	-0.19	0.19
>5.72	82 (25.87)	0.08	0.61	0.09	0.57
Trend			0.78		0.77

BMI-Body Mass Index
NSAID- Non-steroidal anti-inflammatory
MET-Metabolic Equivalent Task
[a]Coefficients in relation to standardized LINE-1 DNA methylation, adjusted for age and sex
[b]Coefficients in relation to standardized LINE-1 DNA methylation, adjusted for age and sex, and mutually adjusted for each lifestyle exposure
[c]Categories defined separately for men and women by drinking recommendations from Canadian Dietary Guidelines
[d]Either prescription or non-prescription use in the past year

carbon metabolism. Through this mechanism, dietary factors may play a role for one-carbon metabolism functions in the cell (20). Exposures such as alcohol consumption, and smoking have been mechanistically linked to disruptions in one-carbon metabolism through malabsorption of folate, and decreased oxygenation in the cell (4,21).

Inflammation is a biological response from the body's immune system in reaction to harmful stimuli. Despite its role as a key immune response, chronic inflammation can be harmful to the human body, and has been linked to oxidative stress and DNA damage in the genome (5). Oxidative stress can result in damage to the DNA molecule, as well as impact DNA methyltransferase activity in the cell. Prostaglandins are active lipid compounds that are involved in inflammation, and have also been shown to affect DNA methyltransferase activity (5). Dietary habits such as high consumption of fruits and vegetables is linked to reduced inflammation in the literature, and obesity is an established cause of chronic inflammation (22-24). Physical activity and NSAID use may play a role against chronic inflammation (5,6). The mechanisms discussed provide plausibility for these risk factors to affect L1 DNA methylation patterns in humans.

Despite the biological plausibility of the lifestyle factors of interest to influence L1 DNA methylation, as well as the established role of these factors of interest on CRC risk, this study reports null results on the relationship between CRC risk factors and L1 DNA methylation. These results suggest that L1 DNA methylation does not represent an important mechanism relating CRC risk factors to carcinogenesis. Alternatively, L1 DNA methylation in non-specific sites, such as PBLs might represent a better marker of early cancer risk, compared to healthy colon tissue.

Several previous investigations have studied relationships between CRC risk factors and L1 DNA methylation in PBLs, while analysis of relationships in healthy colon tissue are limited (6,7,25-27). PBLs may be more susceptible to methylation variation from lifestyle factors compared to healthy colon tissue, leading to higher variability in methylation (10). Critical processes for methylation maintenance such as

detoxification are also known to actively occur in PBLs opening the possibility for greater influence on methylation in peripheral blood, compared to colon tissue (11,12). Although this study hypothesized that CRC risk factors may be better associated with methylation values taken directly from the tissue of interest, the current findings suggest that CRC risk factors do not reflect changes in healthy colon tissue methylation. Instead, methylation occurring in PBLs may represent a more important intermediate mechanism relating CRC risk factors to L1 DNA methylation. This is bolstered both by reports of significant relationships between CRC risk factors and L1 DNA methylation in PBLs, as well as studies reporting significant relationships between L1 DNA methylation in PBLs and early cancer risk (28). Current research is limited in the understanding of methylation variability across different sites and cells, however, this is an active field of study, and future analyses may provide improved understanding of L1 methylation in PBLs compared to healthy colon tissue.

This research adds to two previous publications that suggest a lack of association between alcohol consumption, smoking, and BMI with L1 methylation in the healthy colon (7,19). To our knowledge, this is the first study to investigate the potential for NSAID use and fruit and vegetable consumption to affect L1 DNA methylation in healthy colon tissue.

A major strength of this study is the analysis of methylation directly in the tissue of interest, in contrast to investigations using leukocyte tissues as a surrogate measure for tissue-specific methylation. Furthermore, although cross-sectional analyses are often faced with issues of recall bias, selection bias, and reverse causality, these issues were less of a concern for this investigation. Individuals were unaware of their methylation

status at the time of survey, making it impossible for questionnaire answers to be biased by this information. Additionally, selection into the study could not be biased for this reason. Other strengths include low likelihood of information and selection bias, consideration of a comprehensive set of lifestyle exposures and confounders, and analysis of a continuous outcome, which helped mitigate issues related to study power. Limitations include potential misclassification of exposures, particularly NSAID use, as well as potential for residual confounding due to imperfect measurements of remaining exposures. Results were further limited by a one-time measure of L1 DNA methylation, where repeat measures may have allowed for more accurate measurement of the outcome of interest.

Previous analysis suggests L1 DNA methylation in healthy colon tissue is predictive of adenoma risk, consequently there is value in understanding potential risk factors that may decrease methylation in healthy tissue (16,29), as well as the potential for L1 DNA methylation to serve as an early biomarker of cancer risk (1). Specifically, decreased L1 DNA methylation leading to carcinogenesis can be seen as modifiable intermediate mechanism within the larger relationship between CRC risk factors and cancer. Colorectal cancer is the 2nd most common cancer diagnosis in Canada, accounting for approximately 13% of all diagnoses (30). Screening programs which detect precancerous adenomatous polyps are in place across Canada and have slightly reduced CRC incidence since 2000 (30). However, continuing research that may help identify causes of CRC is integral to future progress.

This study builds on current research relating to lifestyle risk factors associated with CRC and methylation by studying differences in L1 DNA methylation in healthy

colon mucosal tissue. This analysis assessed associations in a more comprehensive set of CRC risk factors than previous research, and facilitated improved understanding of mechanisms relating lifestyle factors to CRC risk.

The lack of association between CRC risk factors and cancer found in this study suggests that CRC risk factors may lead to CRC through alternate mechanisms such as methylation in PBLS, or in other sites and tissues that are currently uncharacterized.

The importance of these results stem from the sparse literature in this field, as well as their potential to direct future studies on the mechanisms involved in leading to CRC. Research studying L1 DNA methylation as a modifiable risk factor in carcinogenesis has implications for targeted cancer prevention, both through genotyping individuals for higher risk of aberrant methylation, and through direct consideration of their current lifestyles.

4.6 References

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Chapter 5

Colorectal cancer risk factors and effect modification by one-carbon metabolism related gene variants in relation to LINE-1 DNA methylation

5.1 Abstract

Reduced LINE-1 DNA methylation and one-carbon metabolism-related gene variants are both shown to increase colorectal cancer (CRC) risk. Exposures related to LINE-1 DNA methylation may have a greater effect on methylation levels for individuals already susceptible to reduced methylation due to possession of a genetic variant. When considered together, these effects may impact methylation patterns in individuals beyond what would be expected if effects were purely additive. This research examines whether genes involved in one-carbon metabolism modify relationships between established CRC risk factors and LINE-1 DNA methylation in healthy colon tissue. This study comprised of 317 individuals scheduled for a routine colonoscopy. Multivariable linear regression was used to study the associations of alcohol consumption, smoking, body mass index, non-steroidal anti-inflammatory drug use, physical activity, and fruit and vegetable consumption with LINE-1 DNA methylation. Product terms between *MTHFR* 677C>T (rs1801133), *MTR* 2756A>G (rs1805087), and *MTRR* 66A>G (rs1801394) genotypes with exposures of interest were used to test effect modification by genotype, and statistically significant interactions between alcohol consumption and *MTHFR* and *MTR* genotypes were observed (p-value interaction=0.009, 0.003). These results present novel interaction analysis by gene variants in the relationships between CRC risk factors and

LINE-1 DNA methylation in the healthy colon. Although effects were not consistently in hypothesized directions, they suggest that one-carbon metabolism-related gene status is an important effect modifier in the relationship between alcohol consumption and LINE-1 DNA methylation.

5.2 Introduction

DNA methylation is a widely studied process in the field of epigenetics. Methylation directly controls transcription of the DNA molecule via transfer of a methyl-group to cytosine residues in CpG dinucleotide pairs, and subsequent blocking of transcription activity (1). The majority of CpG sites (80%) are found in repetitive sequences. The most widely studied category of sequences is L1 (long interspersed nuclear element-1), which comprise approximately 17% of the human genome (2). L1 DNA methylation patterns are modifiable over longer time periods due to environmental and lifestyle exposures, but are shown to be stable in studies measuring variation week-to-week, or even month-to-month (3,4). Hypomethylation of L1 repeats is associated with genomic instability and is associated with increased risk of colorectal cancer (CRC), as well as other cancer sites, including head and neck, stomach, ovarian, cervical, lung, and liver cancers (5-12). These established relationships support the study of L1 DNA methylation as an intermediate endpoint to carcinogenesis in the colon.

CRC is the third most common cancer diagnosis worldwide, and is a cause of significant morbidity and mortality in North America (13). Colorectal tumour development follows an established sequence from aberrant proliferative epithelial dysplasia, to colorectal adenomatous polyps (adenoma), to CRC (14). Screening programs which detect precancerous adenomatous polyps are in place across Canada and

have slightly reduced CRC incidence since 2000 (15). However, continuing research that may help identify causes of CRC is integral to future progress.

The associations between alcohol consumption, smoking, body mass index (BMI), physical activity, non-steroidal anti-inflammatory (NSAID) use, fruit and vegetable intake, and CRC are recognized. Previous research on biologically plausible mechanisms relating listed exposures of interest to L1 DNA methylation therefore supports further study of these relationships as intermediate processes on the pathway to carcinogenesis (16-26).

One-carbon metabolism represents a major mechanism through which the exposures of interest may be linked to aberrant L1 DNA methylation. The primary methyl donor for DNA methylation is S-adenosylmethionine, which is generated via one-carbon metabolism, making one-carbon metabolism a key process in facilitating L1 DNA methylation in the cell (27). Alcohol consumption has also been mechanistically linked to disruptions of the availability of folate in the body through intestinal malabsorption of folate, inhibition of important enzymes involved in one-carbon metabolism reactions, and the cleavage of folate by alcohol metabolites (28). Smoking is known to lead to hypoxia, which decreases oxygenation in the cell. Hypoxia directly affects methionine adenosyltransferase, which synthesizes SAM (29). This is a mechanism by which smoking can directly disrupt one-carbon metabolism and lead to aberrant L1 DNA methylation.

Inflammation represents another mechanism through which exposures of interest can affect L1 DNA methylation. There is some evidence that a pro-inflammatory cytokine - interleukin (IL-6) - may lead to hypomethylation of L1 regions (30). Oxidative

stress is a by-product of chronic inflammation, and can lead to DNA damage, which may indirectly affect L1 DNA methylation (31,32). Dietary habits such as high consumption of fruits and vegetables is linked to reduced inflammation, and obesity is an established cause of chronic inflammation (33-35). Physical activity and NSAID use may be protective against chronic inflammation (36,37).

MTHFR, *MTR*, and *MTRR* are genes that code for enzymes involved in one-carbon metabolism. *MTHFR* codes the enzyme MTHFR, which is responsible for converting dietary folate into 5-methylenetetrahydrofolate, the primary form required for 1-carbon metabolism (27). The *MTR* gene codes MTR, which aids in the production of SAM, a key component of 1-carbon metabolism (27). *MTRR* is responsible for maintaining MTR in its active form through production of methionine synthase reductase (27). Variant alleles in these genes can lead to lower levels of DNA methylation in portions of the genome that are typically methylated via reduced enzyme activity. However, complete functional understanding of the role of specific polymorphisms on DNA methylation remains incomplete (27). When coupled with exposures known to impact L1 DNA methylation, a polymorphism in one of these genes may lead to reduced methylation beyond what would be expected if these effects were purely additive. For example, healthy lifestyle behaviors may have a greater importance in maintaining normal methylation levels for individuals with a variant allele for one of the genes of interest, making it critical to assess their potential role as effect modifiers.

L1 DNA methylation and its role in cancer development has stimulated considerable research in recent years (38,39) (40). However, the majority of these studies analyze tissue samples taken from blood, or adenoma/CRC tissue instead of using healthy

colon tissue.(17,18,22,41-44) Studies that have examined methylation in blood are not ideal as blood is not the target tissue of interest, and methylation measures in blood only moderately correlate with measures in colon tissue (45). Studies focusing on DNA methylation in adenoma or CRC tissue are limited as aberrant methylation in this tissue may be a consequence rather than a cause of disease.

The objective of this study is to determine if the relationships between CRC risk factors and L1 DNA methylation are modified by gene variants associated with one-carbon metabolism in healthy colon tissue.

We hypothesized that alcohol consumption, smoking, and high BMI will be associated with reduced methylation, and that those with variant alleles for each genotype will be more susceptible to reduced methylation. We hypothesized that NSAID use, physical activity, and fruit and vegetable consumption preserve methylation levels, and play a more important role in L1 DNA methylation maintenance in those with variant alleles for each genotype.

5.3 Materials and Methods

5.3.1 Study population and design

This research was nested within a previously conducted cross-sectional study investigating L1 DNA methylation and the risk of adenoma in a cancer-free population (46). Patients undergoing a routine colonoscopy at Hotel Dieu Hospital (Kingston, Canada) were recruited between 2009-2012. Patients were between 40-65 years of age. Individuals were screened if they had a positive fecal occult blood test result, a positive family history of CRC in one or more first-degree relatives, or were at average risk for CRC. Patients in which CRC was detected during colonoscopy were excluded from the

study. Additional exclusion criteria included patients with a genetic condition predisposing to CRC, gastrointestinal abnormalities, a diagnosis of inflammatory bowel disease, diagnosis or treatment of any cancer (except for non-melanoma skin cancers), or detection of serrated adenomas, sessile serrated adenomas, or hyperplastic polyps during colonoscopy screening (to ensure a homogeneous outcome for the original study) (46).

Institutional ethics approval was obtained from the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board (File No. 6004521) and all subjects provided informed consent.

5.3.2 Measurement of LINE-1 DNA methylation

Two pinch biopsies of normal-appearing colon mucosal tissue were collected during the procedure, along with a fasting blood sample. Laboratory methods for obtaining final methylation measures are published elsewhere (46). In brief, DNA was isolated from tissue samples using the 5-PRIME DNA isolation kit (Inter Medico, Markham, Canada). Pinch biopsy samples were combined during DNA extraction. Next, DNA concentrations were measured using Quant-iT™ PicoGreen®, and stored at −20°C. DNA was then bisulfite converted in order to differentiate methylated cytosines from unmethylated cytosines, and PCR was carried out using primers designed for the L1 consensus promoter region sequence (GenBank #: X58075), replicating 8 CpG sites representative of L1 DNA methylation across the genome (47).

High-resolution melt curve (HRMC) analysis was used to quantify L1 loci-specific methylation values. HRMC analysis is a real-time fluorescence-based polymerase chain reaction, and the application of this method to quantify L1 DNA methylation has been described and validated elsewhere (48). This approach allowed for

quantification of average methylation across the 8 CpG sites for each participant. All PCR samples were run in triplicate, and methylation values were averaged across all triplicates and mucosal cell-types.

5.3.3 Genotyping

Leukocyte cell DNA was used to genotype *MTHFR* 677C>T (rs1801133), *MTR* 2756A>G (rs1805087), and *MTRR* 66A>G (rs1801394) using MassARRAY SNP assays at McGill University and the Genome Québec Innovation Centre, Montréal, Canada (49). Differences between observed and expected genotype frequencies under the assumption of Hardy-Weinberg Equilibrium (HWE) were tested using χ^2. Homozygous and heterozygous variant genotypes were combined to maximize study power.

5.3.4 Exposure and confounder measurement

Demographic and lifestyle exposure information was obtained from a self-administered questionnaire completed prior to the colonoscopy appointment. Information on alcohol consumption, smoking, BMI, NSAID use, physical activity, and fruit and vegetable consumption was obtained through the questionnaire. Each exposure was carefully considered in various representations before moving forward with multivariable linear regression.

Participants were asked about their alcohol consumption as a weekly average, representative of consumption over the past year for beer, wine, liquor, and coolers. Average consumption over the past week of each beverage was used to calculate an average for grams of alcohol consumed per day. Categories of consumption were based on sex-specific guidelines outlined by sex-specific guidelines published Canadian Guidelines on Substance Abuse and Addiction in 2017. In men and women, “light”

consumers were those who consumed no more than 2 drinks per day on average, "moderate" consumers were those consuming 3-4 drinks per day, and "heavy" consumers were those consuming 5 or more drinks per day. In women, "light" consumers were those having 1 or fewer drinks per day, "moderate" consumers were those having 2-3 drinks per day, and "heavy" consumers were those having 4 or more drinks per day, on average. Due to the lack of heavy consumers in the dataset (N=3), moderate and heavy consumption categories were combined.

Smoking status was defined as 'never', 'previous', and 'current' for analysis. Participants were classified as a current smoker if they had smoked at least one cigarette a day for the past year, and as a previous smoker if they had smoked at least one cigarette a day for six or more months during any point in their lives.

Information on BMI was calculated from clinical chart reports, and categorized based on established cut-points (50). "Underweight" and "normal weight" categories were combined, as only one individual in the dataset was underweight. Individuals with a BMI below 25 were classified as "normal BMI", individuals with a BMI between 25 and 30 were classified as "overweight BMI", and individuals with a BMI at or above 30 were classified as "obese BMI" (50).

NSAID use was collected as the number of months of continuous use (at least 4 times per week) in the past year for prescription or non-prescription NSAIDs. Due to a lack of homogeneity in categorizing NSAID use in the literature, categories assumed to best summarize use were created based on the distribution of months of use, and initial bivariate analysis (37,51). Number of months of continuous use in the past year of either prescription or non-prescription NSAIDs was used to categorize use into "no use", "less

than 1 year of use", or "1 year of use". The highest consumption category was conceptualized as incorporating individuals who have likely been consuming NSAIDs consistently for greater than one year, but had only reported a maximum of 12 months of use in the past year. Different variable classification by prescription and non-prescription NSAIDs did not point to statistically significant differences in initial analysis (data not shown).

An adapted version of the short-form International Physical Activity Questionnaire (IPAQ-SF) was used to measure physical activity (52). This questionnaire is widely used for the assessment of physical activity and measures average physical activity levels for an individual over the course of a week in the past year (53). Activity is divided into vigorous, moderate, and light, and reported as days per week, and hours per day. Final activity was represented as Metabolic Equivalent of Task (MET) minutes, and divided into quartiles (54).

Information on dietary patterns was obtained through a section of the questionnaire adapted from the Abbreviated Dietary Interview, which covered frequency consumption of 42 common fruits and vegetables per week, over the past year (55). This frequency was summed and averaged to obtain a final measure of frequency of consumption per day, and then categorized into approximately equal quartiles.

Confounders considered in analyses included age, sex, ethnicity, serum-folate, and serum-B_{12}. Age and sex were determined through patient reports, and ethnicity was self-reported and categorized as "Caucasian" or "Other." Serum-folate and B_{12} levels were collected from lab results at Hotel Dieu Hospital. The selected potential

confounders are predictors of L1 DNA methylation and commonly adjusted for in similar analyses (36,56-59).

5.3.5 Statistical Analysis

L1 DNA methylation values were standardized for analyses. Multivariable linear regression was used to assess effect modification by one-carbon metabolism gene variants on the relationship between alcohol consumption, smoking, BMI, NSAID use, physical activity, and fruit and vegetable consumption with L1 DNA methylation in healthy colon tissue. The relationships between CRC risk factors and L1 DNA methylation were modeled separately for each gene. Interactions were evaluated at a predetermined alpha of 0.05. Age and sex were forced into the models as established predictors of L1 DNA methylation, while ethnicity, and serum levels of folate and B_{12} were evaluated as potential confounders using a backwards elimination approach (liberal p-value of 0.15) (60,61). Stratified coefficients and p-values by genotype for each variable of interest were extracted from the same model using contrast terms in SAS 9.4.

5.3.6 Sensitivity Analysis

Our study population included individuals with and without a colorectal adenoma, and a presence of adenoma may have influenced LINE-1 DNA methylation. Therefore, we tested for effect modification by detection of adenoma. Non-significant p-values would suggest that when results are stratified by adenoma cases, the results of our main effects are not significantly different.

5.4 Results

L1 DNA methylation was normally distributed (μ=88.17, σ=3.62). Characteristics of the study population are described in Table 5-1, along with the relationships with L1

DNA methylation, adjusted for age and sex. Briefly, 93% of the population was Caucasian, 57% was female, and 59% were between the ages of 50-60. About one third of the study population had an adenoma at the time of colonoscopy (34%). Females had lower methylation values than males (p=0.001), while age was not significantly related to L1 DNA methylation (p=0.15).

Results from χ^2 tests to determine deviation from HWE frequencies revealed that all genotypes were in HWE, aside from *MTHFR* (χ^2 p-value=0.04).

Results for interaction analysis by one-carbon metabolism gene variants on CRC risk factors and L1 DNA methylation are presented in Tables 5-2 to 5-4. *MTHFR* and *MTR* genotypes revealed significant effect modification for alcohol consumption, while there was no significant interaction by *MTRR* on any CRC risk factors of interest and L1 DNA methylation.

The relationship between alcohol consumption and L1 DNA methylation was significantly modified by the variant allele of *MTHFR* (p=0.009) (Table 5-2). In stratified analysis for individuals with a variant allele for *MTHFR*, light consumers had reduced L1 DNA methylation by 0.64 of a standard deviation compared to abstainers (p=0.002) (Table 2). Moderate consumers also had decreased methylation, however, this relationship was not statistically significant. This trend towards decreasing methylation was not observed in individuals homozygous for the wild-type allele of *MTHFR*. In this group, L1 methylation was higher in both light and moderate consumers compared to abstainers, although these relationships did not reach statistical significance (p=0.11, p=0.06) (Table 5-2). No other interactions were reported by the *MTHFR* genotype.

The relationship between alcohol consumption and L1 DNA methylation was also modified by the variant allele of *MTR* (p=0.003) (Table 5-3). In individuals possessing a variant allele for *MTR*, moderate consumers had increased L1 DNA methylation by 0.75 of a standard deviation compared to abstainers (p=0.004) (Table 5-3). Individuals homozygous for the wild-type allele showed a trend towards reduced methylation in both light and moderate categories, with effects approaching statistical significance (p=0.06, p=0.07) (Table 5-3). No other relationships between CRC risk factors and L1 DNA methylation were modified by the variant allele of *MTR*.

Although interactions by NSAID use were not significant across all genotypes of interest, some effects between categories of NSAID use were significant upon stratification by variant allele. For those with a variant allele of *MTHFR*, short-term users of NSAIDs had decreased methylation by half a standard deviation compared to those in the "no use" category (p=0.003, p interaction=0.17) (Table 5-2). In those with a variant allele of *MTR*, long-term users had significantly increased L1 DNA methylation by 0.43 of a standard deviation, compared to those in the "no use" category (p=0.04, p interaction=0.20) (Table 5-3). Although overall interaction values were not significant for these effects, there appears to be a pattern of decreased methylation in short-term users, and increased methylation in long-term users in this data.

To assess if our results were robust to the inclusion of subjects that had a colorectal adenoma, we tested for interaction by adenoma in our analysis of the relationships between alcohol consumption, and smoking with LINE-1 DNA methylation, stratified by allele variants for all three genotypes. P-values were highly

Table 5-1. The association of study population characteristics and genotypes with LINE-1 DNA methylation in colon tissue (N=317)

Characteristic	N (%)	Coefficient[a]	p-value
Sex			0.001
Male	137 (43.22)	Ref.	
Female	180 (56.78)	-0.37	
Age			0.15
39-50	55 (17.10)	Ref.	
50-60	184 (58.39)	-0.03	0.86
60+	78 (24.52)	0.23	0.19
Adenoma			0.19
No	209 (65.93)	Ref.	
Yes	108 (34.07)	-0.16	
Ethnicity			0.78
Caucasian	295 (93.44)	Ref.	
Other	22 (6.56)	0.06	
MTHFR			0.29
CC	153 (48.26)	Ref.	
CT/TT	164 (51.73)	0.11	
MTR			0.55
AA	199 (62.78)	Ref.	
AG/GG	118 (37.22)	-0.07	
MTRR			0.97
AA	61 (19.14)	Ref.	
AG/GG	256 (80.75)	0.005	

[a]Standardized LINE-1 DNA methylation coefficients, age and sex adjusted.

non-significant (Appendix Tables B-3 to B-5), and suggest that our findings are robust to the inclusion of subjects with an adenoma.

5.5 Discussion

Decreased methylation of L1 elements is an established mechanism in carcinogenic processes, however research on lifestyle and genetic factors that may impact early hypomethylation in healthy tissues is limited (40).

Leukocytes are a convenient and popular tissue of choice for the analysis of DNA methylation, however, DNA methylation patterns are tissue-specific, and methylation patterns found in blood samples may not reflect methylation occurring in tissues of interest, such as colon mucosal tissue. Analysis of L1 DNA methylation in blood tissues compared to tissues of interest reveal weak correlations in methylation patterns between the two types (45) (62). In addition, variability in methylation values found in blood may be a function of the type of cells chosen for analysis, rather than person-to-person variability due to exposures (63). These limitations reinforce the importance of analysis of L1 DNA methylation in the tissue of interest.

This study assessed interaction by one-carbon metabolism gene variants on the relationship between CRC risk factors and L1 DNA methylation in healthy colon tissue samples. Results from this analysis are not consistent across categories of exposure, but provide novel evidence for a significant interaction by *MTHFR* and *MTR* genotypes on the relationship between alcohol consumption and L1 DNA methylation in healthy colon tissue. We found that in individuals with a variant allele for *MTHFR*, those that were light consumers had significantly decreased L1 DNA methylation compared to abstainers, and that moderate drinkers had reduced methylation that did not reach statistical significance. However, the reverse was found for the *MTR* genotype, in which

individuals with a variant allele and moderate consumption had significantly increased L1 DNA methylation compared to abstainers.

Alcohol consumption is an established risk factor for several cancer sites (64). Alcohol consumption may lead to decreased L1 DNA methylation through disruption of one-carbon metabolism. Specifically, alcohol reduces folate availability in the body through intestinal malabsorption, inhibition of important enzymes involved in one-carbon metabolism, and the cleavage of folate by alcohol metabolites - processes that are essential to the functioning of one-carbon metabolism (28). *MTR* is a commonly studied gene influencing one-carbon metabolism, and in-vitro studies support the importance of *MTR* functioning in the one-carbon metabolism cycle (65). The variant allele *G* is associated with reduced one-carbon metabolism efficiency, and increased risk of colorectal cancer (27,66,67). However, functional data on this polymorphism is limited, and some studies present decreased risk of cancer and increased serum folate levels associated with the *G* allele at position 2576 for *MTR* (68,69).

It is possible that limited variability in alcohol consumption affected complete analysis of the relationship between consumption and L1 DNA methylation. If cancer risk related to alcohol consumption is U-shaped, it is possible that these effects may also be expected to differ in the relationships of interest, making the lack of heavy consumers partially responsible for the effects observed. However, current studies suggest that although the relationship between alcohol consumption and cardiovascular disease follows a U-shape, increased risk of CRC has been observed in even light drinkers (70). Another potential difficulty in the interpretation of these results stems from the fact that any observed differences between *MTR* genotypes may be due to linkage disequilibrium by another

polymorphism with opposing effects in the same gene. However, to our knowledge, no other polymorphisms have been identified in this gene (71).

Despite the opposing effects by *MTHFR* and *MTR* genotypes, statistically significant interactions were observed for both genotypes in relation to alcohol consumption. A statistically significant relationship between alcohol consumption and L1 DNA methylation in healthy colon tissue has not been previously reported. It is important not aware of their methylation levels when recalling exposures, and any misclassification would be non-differential – attenuating our results to the null. In addition, despite having measured several potential confounders, residual confounding was also likely present due to imprecise measurement, such as a lack of information on intensity of smoking, or NSAID dose. There are likely several determinants of L1 DNA methylation that are unknown, and therefore uncontrolled confounding from these unmeasured variables is also possible. Another potential limitation is the lack of HWE for the *MTHFR* genotype. However, this would not have impacted our effect modification analysis, and was arguably expected considering the population under study was a non-representative screening population.

Despite the cross-sectional design, it is doubtful that L1 DNA methylation could influence exposure to the lifestyle factors of interest, making reverse-causality unlikely. Information and selection biases were also mitigated due to objective outcome measurement. Additionally, we considered a comprehensive set of potential confounders in our statistical models, limiting uncontrolled confounding. Although two previous analyses have addressed a partial set of CRC risk factors in relation to L1 DNA methylation, to our knowledge no previous publications have studied effect modification

Table 5-2. The interaction of *MTHFR* with CRC lifestyle exposures and LINE-1 DNA methylation in healthy colon tissue (N=317)

		MTHFR (CC)			***MTHFR*** (CT/TT)		
Lifestyle Exposure	**p-value***	**N (%)**	**Coefficient[a]**	**p-value**	**N (%)**	**Coefficient[a]**	**p-value**
Alcohol Consumption[b]							
Abstainers	0.13	21 (6.62)	Ref.		25 (7.89)	Ref.	
Light		97 (30.59)	0.37	0.11	105 (33.12)	-0.64	0.002
Moderate		35 (11.04)	0.50	0.06	34 (10.73)	-0.25	0.32
p-value interaction=0.009							
Smoking	0.09						
Never		84 (26.50)	Ref.		86 (27.13)	Ref.	
Former		53 (16.72)	-0.06	0.71	53 (16.72)	-0.12	0.48
Current		16 (5.05)	0.06	0.81	25 (7.89)	0.43	0.04
p-value interaction=0.50							
BMI	0.40						
Underweight/Normal		55 (17.35)	Ref.		52 (16.40)	Ref.	
Overweight		54 (17.03)	0.007	0.97	62 (19.56)	-0.09	0.62
Obese		44 (13.88)	0.21	0.29	50 (15.77)	0.15	0.41
p-value interaction=0.94							
NSAID Use[c]	0.15						
No use		101 (31.86)	Ref.		105 (33.12)	Ref.	
Less than 1 year of use (short-		23 (7.26)	0.02	0.92	24 (7.57)	-0.47	0.03

term)							
Full year of use (long-term)		29 (9.15)	0.37	0.07	35 (11.04)	-0.05	0.80
p-value interaction=0.17							
Physical Activity (MET minutes per week)	0.11						
<1908		35 (11.04)	Ref.		48 (15.14)	Ref.	
1908-3745		39 (12.30)	0.27	0.23	39 (12.30)	-0.20	0.35
3745-6542		35 (11.04)	-0.18	0.43	44 (13.88)	-0.11	0.57
>6542		44 (13.88)	0.30	0.17	33 (10.41)	0.23	0.27
p-value interaction=0.36							
Fruit and Vegetable Consumption (number of servings per day)	0.23						
<2.9		38 (11.99)	Ref.		41 (12.93)	Ref	
2.9-4.1		31 (9.78)	-0.03	0.90	48 (15.14)	0.24	0.23
4.1-5.72		39 (12.30)	-0.42	0.05	38 (11.99)	0.06	0.77
>5.72		45 (14.20)	0.08	0.72	37 (11.67)	0.11	0.59
p-value interaction=0.41							

BMI-Body Mass Index, NSAID- Non-steroidal anti-inflammatory drug, MET-Metabolic Equivalent Task
*p-value for main-effect relationship between exposure and LINE-1 DNA methylation, adjusted for age and sex
[a]Standardized LINE-1 DNA methylation adjusted for age and sex, and mutually adjusted for each lifestyle exposure
[b]Categories defined separately for men and women by drinking recommendations from Canadian Guidelines on Substance Abuse and Addiction.
[c]Either prescription or non-prescription use in the past year.

Table 5-3. The interaction of *MTR* with CRC lifestyle exposures and LINE-1 DNA methylation in healthy colon tissue (N=317)

Lifestyle Exposure	p-value*	*MTR* (AA) N (%)	Coefficient[a]	p-value	*MTR* (AG/GG) N (%)	Coefficient[a]	p-value
Alcohol Consumption[b]	0.13						
Abstainers		23 (7.25)	Ref.		23 (7.25)	Ref.	
Light		135 (42.58)	-0.40	0.06	67 (21.13)	-0.02	0.94
Moderate		41 (12.93)	-0.45	0.07	28 (8.83)	0.75	0.004
p-value interaction=0.003							
Smoking	0.09						
Never		114 (35.96)	Ref.		56 (17.66)	Ref.	
Former		60 (18.92)	-0.03	0.85	46 (14.51)	-0.38	0.05
Current		25 (7.88)	0.16	0.43	16 (5.04)	0.32	0.22
p-value interaction=0.27							
BMI	0.40						
Underweight/Normal		71 (22.39)	Ref.		36 (11.35)	Ref.	
Overweight		74 (23.34)	-0.16	0.30	42 (13.25)	0.16	0.45
Obese		54 (17.03)	0.14	0.41	40 (12.61)	0.29	0.20
p-value interaction=0.50							
NSAID Use[c]	0.15						
No use		130 (41.01)	Ref.		76 (23.97)	Ref.	
Less than one year of use (short-term)		30 (9.46)	-0.18	0.35	17 (5.36)	-0.20	0.44

Full year of use (long-term)		39 (12.30)	-0.07	0.66	25 (7.89)	0.43	0.04
p-value interaction=0.20							
Physical Activity (MET minutes per week)	0.11						
<1908		53 (16.72)	Ref.		30 (9.46)	Ref.	
1908-3745		46 (14.51)	0.08	0.69	32 (10.09)	0.10	0.68
3745-6542		52 (16.40)	-0.21	0.26	27 (8.52)	0.21	0.42
>6542		48 (15.14)	0.30	0.12	29 (9.15)	0.23	0.34
p-value interaction=0.48							
Fruit and Vegetable Consumption (number of servings per day)	0.23						
<2.9		47 (14.83)	Ref.		32 (10.09)	Ref	
2.9-4.1		54 (17.03)	0.01	0.90	25 (7.89)	0.35	0.16
4.1-5.72		45 (14.20)	-0.08	0.68	32 (10.09)	-0.34	0.13
>5.72		53 (16.72)	0.29	0.12	29 (9.15)	-0.12	0.64
p-value interaction=0.12							

BMI-Body Mass Index, NSAID- Non-steroidal anti-inflammatory drug, MET-Metabolic Equivalent Task
*p-value for main effect relationship between exposure and LINE-1 DNA methylation, adjusted for age and sex
[a]Standardized LINE-1 DNA methylation adjusted for age and sex, and mutually adjusted for each lifestyle exposure
[b]Categories defined separately for men and women by drinking recommendations from Canadian Guidelines on Substance Abuse and Addiction.
[c]Either prescription or non-prescription use in the past year.

Table 5-4. The interaction of *MTRR* with CRC lifestyle exposures and LINE-1 DNA methylation in healthy colon tissue (N=317)

		MTRR (AA)			***MTRR*** (AG/GG)		
Lifestyle Exposure	**p-value***	**N (%)**	**Coefficient[a]**	**p-value**	**N (%)**	**Coefficient[a]**	**p-value**
Alcohol Consumption[b]	0.13						
Abstainers		8 (2.52)	Ref.		38 (11.99)	Ref.	
Light		38 (11.99)	0.29	0.45	164 (51.74)	-0.24	0.15
Moderate		15 (4.73)	0.71	0.11	54 (17.03)	-0.09	0.65
p-value interaction=0.29							
Smoking	0.09						
Never		33 (10.41)	Ref.		137 (43.22)	Ref.	
Former		22 (6.94)	-0.57	0.05	84 (26.50)	-0.12	0.36
Current		6 (1.89)	0.02	0.97	35 (11.04)	0.24	0.18
p-value interaction=0.43							
BMI	0.40						
Underweight/Normal		20 (6.31)	Ref.		87 (27.44)	Ref.	
Overweight		26 (8.20)	-0.04	0.89	90 (28.39)	-0.01	0.96
Obese		15 (4.73)	0.53	0.14	79 (24.92)	0.12	0.41
p-value interaction=0.47							
NSAID Use[c]	0.15						
No use		38 (11.99)	Ref.		168 (53.00)	Ref.	
Less than one year of use		8 (2.52)	-0.71	0.11	39 (12.30)	-0.17	0.32

(short-term)							
Full year of use (long-term)		15 (4.73)	0.39	0.19	49 (15.46)	0.12	0.41
p-value interaction=0.30							
Physical Activity (MET minutes per week)	0.11						
<1908		11 (3.84)	Ref.		72 (22.71)	Ref.	
1908-3745		19 (5.99)	0.36	0.39	59 (18.61)	0.03	0.88
3745-6542		12 (3.79)	0.84	0.11	67 (21.14)	-0.22	0.17
>6542		19 (5.99)	0.79	0.05	58 (18.30)	0.16	0.34
p-value interaction=0.26							
Fruit and Vegetable Consumption (number of servings per day)	0.23						
<2.9		15 (4.73)	Ref.		64 (20.19)	Ref	
2.9-4.1		13 (4.10)	0.43	0.25	66 (20.82)	0.04	0.82
4.1-5.72		16 (5.05)	-0.28	0.50	61 (19.24)	-0.28	0.09
>5.72		17 (5.36)	0.05	0.89	65 (20.50)	0.10	0.56
p-value interaction=0.73							

BMI-Body Mass Index, NSAID- Non-steroidal anti-inflammatory drug, MET-Metabolic Equivalent Task
*p-value for main effect relationship between exposure and LINE-1 DNA methylation, adjusted for age and sex
[a]Standardized LINE-1 DNA methylation adjusted for age and sex, and mutually adjusted for each lifestyle exposure
[b]Categories defined separately for men and women by drinking recommendations from Canadian Guidelines on Substance Abuse and Addiction.
[c]Either prescription or non-prescription use in the past year.

by one-carbon metabolism genes on the relationships of interest in healthy colon tissue (78,79).

5.6 Conclusion

Overall, this research provides evidence that one-carbon metabolism genes are important effect modifiers of the relationship between alcohol consumption and L1 DNA methylation. The novel interaction analyses by one-carbon metabolism genes on the relationships between CRC risk factors and L1 DNA methylation contribute to the understanding of how known cancer risk factors may influence methylation changes in the healthy colon. The results indicate the relationship between alcohol consumption and L1 DNA methylation is modified by *MTHFR* and *MTR* gene variants. These findings have implications for personalized cancer prevention upon further validation in larger study designs. Underlying biological mechanisms behind these interactions also require further investigation.

5.7 References

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Chapter 6

Discussion

6.1 Summary of Findings

In main effects analysis, colorectal cancer (CRC) risk factors, including alcohol consumption, smoking, body mass index (BMI), non-steroidal anti-inflammatory (NSAID) use, physical activity, and fruit and vegetable consumption were not related to Long interspersed nuclear element-1 (L1) DNA methylation in healthy colon tissue from a screening population. Interaction analysis by one-carbon metabolism gene variants on the relationships between CRC risk factors and L1 DNA methylation revealed effect modification by *MTHFR* and *MTR* genes on the relationship between alcohol consumption and L1 DNA methylation. However, all relationships were not in hypothesized directions. In individuals with a variant allele for *MTHFR*, those that were light consumers had significantly decreased L1 DNA methylation compared to abstainers (β=-0.64, p-value=0.002). However, the reverse was found for the *MTR* genotype, in which individuals with a variant allele and moderate consumption had significantly increased L1 DNA methylation compared to abstainers (β=0.75, p-value=0.004).

6.2 Limitations

This research was undertaken using data from a cross-sectional study examining DNA methylation in relation to risk for prevalent adenomas. DNA methylation was measured in healthy tissue biopsies taken from the descending colon 10 cm away from any lesion or abnormality. Limitations in this analysis stem from the epidemiologic

design, as well as the specific measurement and interpretation of L1 DNA methylation data.

6.2.1 Methodological Limitations of Study Design

Foremost, cross-sectional measures opened up the possibility for exposure misclassification, as specific relevant time windows remain unclear in relation to L1 DNA methylation for several exposures of interest. However, this study sought to evaluate lifestyle exposures that should remain consistent overtime. For example, drinking, smoking, BMI, and physical activity, and diet are exposures that are habitual, and likely consistent in the cohort of individuals aged 40 or above, and this may have mitigated some issues related to distinguishing the relevant time window of exposure.

Some epidemiologic limitations of analysis were due to sample size. However, these issues were somewhat mitigated by the investigation of a continuous outcome and reasonable distributions of the exposure of interest. Still, the ability to detect interactions between genotypes and CRC risk factors was limited, especially considering the minimal detectable effect sizes calculated for main effects (Chapter 2, Table 3-2). Another limitation due to sample size was the combining of heterozygous and homozygous variant genotypes. Frequencies of homozygous variant genotypes were not otherwise predicted to be high enough for comparisons to homozygous normal genotypes. Unfortunately, information on heterozygous genotypes was limited in the literature, and it was not possible to determine if combination of heterozygous and homozygous variant genotypes is appropriate given allele combination phenotypes (1). It is possible that differences between some genotypes were not detected for this reason.

Although cross-sectional analyses are often faced with issues of recall bias, selection bias, and reverse causality, these issues were less of a concern for this investigation. Individuals were unaware of their methylation status at the time of survey, making it impossible for questionnaire answers to be biased by this information. Additionally, selection into the study could not be biased for this reason. Reverse causality is also unlikely for the relationships studied, as it would imply that L1 DNA methylation causes exposure to the lifestyle factors of interest. This is unlikely for factors such as alcohol consumption, smoking, physical activity, NSAID use, or fruit and vegetable consumption. However, there is some evidence that the epigenome has the propensity to impact onset of chronic adult diseases, such as obesity (2,3). Although this research is relatively new, it makes it difficult to rule out the potential for reverse causality between a relationship between BMI and L1 DNA methylation. Although this research did not find any significant relationships between BMI and L1 DNA methylation, the potential for reverse causality warrants careful interpretation of significant results in future studies.

Another consideration of methylation analysis is the role of age as a potential confounder in this study. Although all modeling was conducted while adjusted for age, it is possible that age may represent a cumulative lifestyle effect of the risk factors of interest on L1 DNA methylation. Removal of age from modeling did not affect the results of this thesis, however, future reports should consider the effect of these lifestyle factors through age, and consider this role in modeling approaches.

6.2.2 Multiple Comparisons

This analysis consisted of multiple comparisons between several exposures of interest, and the outcome variable, as well as studying interactions between each exposure and three gene variants with L1 DNA methylation. When conducting exploratory analyses without *a-priori* hypotheses, multiple comparisons can increase the likelihood of producing false positive results (4). However, this analysis consisted of specific *a-priori* hypotheses supported by previous studies grounded in biologically plausible mechanisms. Therefore, adjustment for multiple comparisons in analysis was not deemed necessary.

6.2.3 Methodological Limitations of Outcome Measurement

Measurement of L1 DNA methylation introduced potential limitations in analysis. Foremost, tissues were sampled at a single point in time, and therefore the outcome was unable to account for possible fluctuations in methylation values. As previously discussed, L1 DNA methylation is considered stable over shorter time-periods (5,6). However, it remains possible that capturing methylation at different time points may have lead to a more accurate outcome assessment. The field of epigenetic epidemiology continues to evolve, and standardized methods for collecting and analyzing tissue methylation are lacking. It is therefore difficult to directly compare absolute methylation values across studies and identify the magnitude of change in methylation required to be of clinical significance. This limits the ability to consider clinical significance following statistically significant results in this thesis. To mitigate this, standardized methylation values were used throughout analysis. However, standardized analysis methods and

prospective cohort studies are key to better interpretation of the clinical significance of absolute changes in L1 DNA methylation.

In addition, we were unable to control for cell-type distribution in collected samples. It is therefore possible that our results reflect changes in cell type rather than changes in DNA methylation (7). A study specifically evaluating L1 DNA methylation across different cell types in the colon, including epithelial, connective, and muscle tissue, found statistically significant differences in mean methylation values for connective and muscle tissue compared to epithelial tissue samples (p-value=0.02, p-value=0.002) (8). Furthermore, research suggests that cell type distribution may be affected by specific lifestyle exposures, mainly dietary supplementation, and vegetable intake. This may have confounded the relationship between dietary exposures and L1 DNA methylation in the current analysis (9,10). Information on the relationship between the remaining exposures of interest and cell type distribution in the colon is limited. Despite this potential limitation, a recent analysis by Zhu et al. found that cell type distribution did not confound the relationship between predictors including age, BMI, smoking, and alcohol consumption in their study (11).

Another limitation linked to DNA methylation is the method used for methylation quantification. High-resolution melt curve analysis targeted 8 CpG sites intended to represent average L1 methylation across the genome. However, this pooled measure restricted analysis within specific genomic regions or chromosomes. Although, the selected sites have been validated in capturing L1 methylation, the current method could not identify methylation happening in regions that may be more susceptible to epigenetic changes from the exposures of interest, or those more relevant to disease risk (12). The

recent development of chip-based approaches in future analyses may help mitigate this concern, as these approaches are able to quantify methylation at single-nucleotide resolution (13). However, traditional PCR based methods use primers specifically designed to capture thousands of repeated regions across the genome, and are not necessarily inferior to newer chip-based technologies. There remains a lack of a gold-standard method for methylation quantification.

Methods of outcome assessment were susceptible to non-differential misclassification due to random error. Sources of random error in outcome measurement included: cross-sectional measurement of methylation, potentially different methylation levels across different mucosal cell types retrieved from biopsy, and location selection for tissue biopsy. The above criticisms represent potential for non-differential misclassification of L1 DNA methylation. It was expected that a continuous outcome measure, and correlated methylation levels from tissue biopsies might mitigate some of these concerns, however it is possible that null results for main effects analyses were partially a result of attenuation to the null due to the described random error.

Finally, the colon tissue biopsies conducted were limited in the cell types captured. For example, biopsies of the colon are typically unable to capture stem cells in deeper tissue layers (14). This would exclude the study of methylation in colon stem cells from analysis, preventing study of how exposures of interest may impact methylation in these cells. However, basic science research points to differential methylation patterns in stem cells, compared to differentiated cells, in which case lack of stem cells in tissue biopsies may be less of a concern (15).

6.3 Strengths

Despite the described limitations, this study had several strengths. Foremost, L1 DNA methylation was measured in healthy colon tissue, in contrast to blood-based measures. Direct measurement in the tissue of interest alleviated the need to assume that peripheral blood leukocytes provide an adequate surrogate measure of methylation in the tissue of interest. Furthermore, as previously described, objective outcome measurement mitigated concerns regarding recall bias, selection bias, and reverse-causality. Although some exposure measures were self-reported, any study biases were likely non-differential because the participants were unaware of their L1 DNA methylation status. Finally, novel analysis of associations between CRC risk factors and interactions by one-carbon metabolism gene variants on the relationship with L1 DNA methylation was conducted. Testing for interaction by adenoma case for each exposure of interest did not reveal significant results for alcohol consumption or smoking among all three genotypes (Appendix B, Table B-3 to B-5), further bolstering the final conclusions drawn from the analysis. This work is the first to elucidate on the strength and direction of these relationships in a main effects analysis, and stratified by one-carbon metabolism related gene variants.

6.4 Generalizability

This study was nested in a previously conducted cross-section analysis. Specific exclusion criteria included: patients in which CRC was detected during colonoscopy, patients with a genetic condition predisposing to CRC, gastrointestinal abnormalities, a diagnosis of inflammatory bowel disease, diagnosis or treatment of any cancer (except for non-melanoma skin cancers), or detection of serrated adenomas, sessile serrated

adenomas, or hyperplastic polyps during colonoscopy screening (16). Due to these extensive exclusion criteria, it was inappropriate to refer the population as a cohort, or a population of cases and controls. Due to the use of a screening population for study objectives, it is expected that distributions of exposures and genetic variants differed from those of the general population. However, the study objectives were oriented towards understanding of biologic relationships, and lack of a representative-cohort was not of concern for this analysis.

6.5 LINE-1 DNA Methylation in Tissues Compared to Peripheral Blood Leukocytes

This thesis sought to determine whether CRC related lifestyle factors may modify L1 DNA methylation patterns in healthy colon tissue. Previous studies assessing this relationship have used PBL tissue methylation as a surrogate for methylation measurement in the colon, and it was hypothesized that healthy colon tissue methylation may represent a superior early marker of CRC risk. However, in main effects analyses there was no significant relationships between each CRC risk factor assessed and L1 DNA methylation in colon tissue. It is possible that methylation occurring in PBLs may represent a more important intermediate mechanism relating CRC risk factors to L1 DNA methylation. This is bolstered both by reports of significant relationships between CRC risk factors and L1 DNA methylation in PBLs, as well as studies reporting significant relationships between L1 DNA methylation in PBLs and early cancer risk (17). A significant relationship between CRC risk factors and L1 DNA methylation in PBLs was also found in the currently studied population, in contrast to the results presented in this thesis (18). Current research is limited in the understanding of methylation variability across different sites and cells, however, this is an active field of study, and future

analyses may provide improved understanding of L1 methylation in PBLs compared to healthy colon tissue.

6.6 Implications and Future Directions

Colorectal cancer is the 2nd most common cancer diagnosis in Canada, accounting for approximately 13% of all diagnoses (19). Screening programs which detect precancerous adenomatous polyps are in place across Canada and have slightly reduced CRC incidence since 2000 (19). However, continuing research that may help identify causes of CRC is integral to future progress.

This study builds on current research relating to lifestyle risk factors associated with CRC and methylation by studying differences in L1 DNA methylation in healthy colon mucosal tissue. The analysis assessed associations in a more comprehensive set of CRC risk factors than previous research, and facilitated improved understanding of early risk factors that may lead to CRC.

Results of the main effects analysis did not point to significant associations between CRC risk factors and L1 DNA methylation in healthy colon tissue. However, when testing for effect modification by one-carbon metabolism gene variants, statistically significant effects were measured for *MTHFR* and *MTR* gene variants on the relationship between alcohol consumption and L1 DNA methylation. Although these effects were not all in the hypothesized directions, they provide novel information regarding the assessed relationships. Although previous literature suggests that alcohol consumption and variant alleles of *MTR* may lead to reduced methylation, some studies have reported effects similar to those observed in this analysis, in which the variant allele of *MTR* was associated with increased gene functioning, and alcohol consumption was associated with

increased global methylation levels (20,21). Biological mechanisms behind the results presented in these previous analyses were not elucidated, and highlight a gap in basic science knowledge regarding the propensity for alcohol consumption and a gene variant of *MTR* to create a synergistic effect leading to increased L1 DNA methylation in colon tissue.

Overall, this research provides information on the relationship between CRC risk factors and L1 DNA methylation, and suggests that *MTHFR* and *MTR* may be important effect modifiers of the relationships studied. Future analyses should consider effect modification by one-carbon metabolism related genes when investigating similar relationships. Considering the novelty of study objectives, and exploratory nature of the interaction analyses, all study results should be replicated in larger investigations before a definitive conclusion on the propensity for CRC risk factors to affect L1 DNA methylation is made.

6.7 Conclusion

Reduced L1 DNA methylation is linked to increased risk of colorectal cancer (22). One-carbon metabolism-related gene variants are also shown to increase CRC risk in individuals with variant alleles. Although CRC risk factors were not related to L1 DNA methylation in the healthy colon, significant effect modification by *MTHFR* and *MTR* gene variants on the relationship between alcohol consumption and L1 DNA methylation was discovered. Results from this study require validation in larger analyses for further clarification of the relationships investigated.

This research has implications for targeted cancer prevention, both through genotyping individuals for higher risk of aberrant methylation, and through direct

consideration of their current lifestyles. The focus of this work on common gene variants implies high population-impact of any significant results, as compared to studies assessing rare gene variants in the context of cancer research.

DNA methylation is modifiable, therefore an understanding of factors impacting methylation are important in their ability to lead to targeted interventions based on lifestyle changes, and dietary supplementation aimed at stabilizing L1 DNA methylation in individuals in which aberrant methylation is identified (23). These targeted interventions are far off in the future, however, they highlight the potential and importance of studying modifiable biomarkers, such as the work presented in this thesis.

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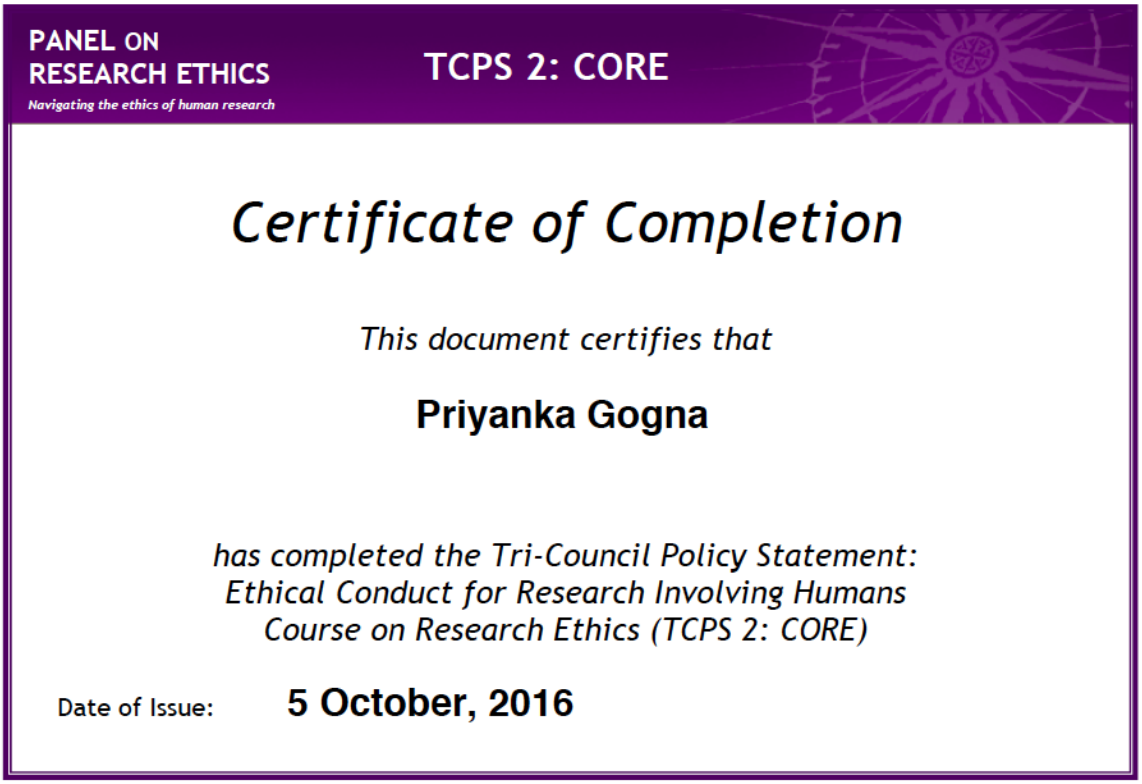
APPENDIX A - Ethics Certificate and Letter of Approval



PANEL ON RESEARCH ETHICS
Navigating the ethics of human research

TCPS 2: CORE

Certificate of Completion

This document certifies that

Priyanka Gogna

has completed the Tri-Council Policy Statement:
Ethical Conduct for Research Involving Humans
Course on Research Ethics (TCPS 2: CORE)

Date of Issue: **5 October, 2016**

QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD (HSREB)

HSREB Initial Ethics Clearance

June 22, 2017

Dr. Will King
Department of Public Health Sciences
Carruthers Hall
Queen's University

ROMEO/TRAQ: #6021193
Department Code: EPID-581-17
Study Title: The Influence of Environmental Risk Factors of Colorectal Cancer and Gene-Environment Interactions on LINE-1 DNA Methylation
Co-Investigators: Ms. P. Gogna
Review Type: Delegated
Date Ethics Clearance Issued: June 22, 2017
Ethics Clearance Expiry Date: June 22, 2018

Dear Dr. King,

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the application and granted ethics clearance for the documents listed below. Ethics clearance is granted until the expiration date noted above.

- Protocol/Thesis Outline

Documents Acknowledged:

- CORE Certificate – P. Gogna

Amendments: No deviations from, or changes to the protocol should be initiated without prior written clearance of an appropriate amendment from the HSREB, except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

Renewals: Prior to the expiration of your ethics clearance you will be reminded to submit your renewal report through ROMEO. Any lapses in ethical clearance will be documented on the renewal form.

Completion/Termination: The HSREB must be notified of the completion or termination of this study through the completion of a renewal report in ROMEO.

Reporting of Serious Adverse Events: Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other serious adverse events

must be reported within 15 days after becoming aware of the information.

Reporting of Complaints: Any complaints made by participants or persons acting on behalf of participants must be reported to the Research Ethics Board within 7 days of becoming aware of the complaint. Note: All documents supplied to participants must have the contact information for the Research Ethics Board. Investigators please note that if your trial is registered by the sponsor, you must take responsibility to ensure that the registration information is accurate and complete.

Yours sincerely,

Allan L. Clark.

Chair, Health Sciences Research Ethics Board

The HSREB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations, Canadian General Standards Board, and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP). Federalwide Assurance Number: FWA#:00004184, IRB#:00001173

HSREB members involved in the research project do not participate in the review, discussion or decision.

APPENDIX B-Supplementary Tables

Table B-1. Linear regression analysis between all variables of interest and LINE-1 DNA methylation

Lifestyle Exposure	N (%)	Coefficient[a]	p-value
Alcohol Consumption[b]			0.13*
Abstainers	46 (14.51)	Ref.	
Light	202 (63.72)	-0.19	0.23
Moderate	69 (21.77)	0.07	0.69
Trend			0.71
Continuous		0.02	0.29
Smoking			0.09*
Never	170 (53.64)	Ref.	
Former	106 (33.44)	-0.16	0.19
Current	41 (53.63)	0.24	0.15
Trend			0.30
BMI			0.40*
Underweight/Normal	107 (29.65)	Ref.	
Overweight	116 (36.59)	-0.02	0.85
Obese	94 (33.75)	0.16	0.26
Trend			0.31
Continuous		0.008	0.80
NSAID Use[c]			0.15*
No use	206 (64.98)	Ref.	
Less than one year of use (short-term)	47 (14.83)	-0.19	0.24
Full year of use (long-term)	64 (20.19)	0.19	0.18
Trend			0.36
Continuous		0.004	0.50
Physical Activity (MET minutes per week)			0.11*
<1908	83 (26.18)	Ref.	
1908-3745	78 (24.61)	-0.007	0.96
3745-6542	79 (24.92)	-0.14	0.34

>6542	77 (24.29)	0.24	0.11
Trend			0.13
Continuous		0.00006	0.21
Fruit and Vegetable Consumption (number of servings per day)			0.23*
<2.9	79 (24.92)	Ref.	
2.9-4.1	79 (24.92)	0.13	0.41
4.1-5.72	77 (24.29)	-0.18	0.25
>5.72	82 (25.87)	0.08	0.61
Trend			0.78
Continuous		0.006	0.93
Sex			0.001
Male	137 (43.22)	Ref.	
Female	180 (56.78)	-0.37	0.001
Age			0.15
39-50	55 (17.10)	Ref.	
50-60	184 (58.39)	-0.03	0.86
60+	78 (24.52)	0.23	0.19
Trend			
Continuous		0.05	0.15
Ethnicity			0.78
Caucasian	295 (93.44)	Ref.	
Other	22 (6.56)	0.06	0.78
Folate			0.56*
<27	80 (25.24)	Ref.	
27-37	79 (24.92)	0.05	0.77
37-45	63 (19.97)	-0.13	0.42
>45	95 (29.97)	0.09	0.53
Trend			0.56
Continuous		0.13	0.78
B12			0.17*

<202	81 (25.55)	Ref.	
202-275	78 (24.61)	-0.31	0.04
275-382	78 (24.61)	-0.03	0.82
>382	80 (25.24)	-0.06	0.71
Trend			0.17
Continuous		0.002	0.96

BMI-Body Mass Index
NSAID- Non-steroidal anti-inflammatory
MET-Metabolic Equivalent Task
[a]Coefficients in relation to standardized LINE-1 DNA methylation, adjusted for age and sex
[b]Categories defined separately for men and women by drinking recommendations from Canadian Dietary Guidelines
[c]Either prescription or non-prescription use in the past year
[*]Quadratic term in regression modeling not significant, p-value from model with quadratic term removed

Table B-0-2. Matrix of relationships between all variables of interest[a]

	Alcohol Consumption[b]	Smoking	Physical Activity	NSAID Use[c]	Fruit and Vegetable Servings	BMI	Sex	Age	Ethnicity	Folate	B12
Alcohol Consumption		x^2=0.09 0.11	x^2=0.13 0.13	x^2=0.24 0.09	x^2=0.04 0.14	x^2=0.15 0.10	x^2=0.59 0.06	x^2=0.24 0.09	x^2=0.49 0.07	x^2=0.02 0.16	x^2=0.10 0.13
Smoking			x^2=0.28 0.10	x^2=0.84 0.08	x^2=0.84 0.06	x^2=0.26 0.09	x^2=0.22 0.09	x^2=0.73 0.06	x^2=0.04 0.14	x^2=0.001 0.18	x^2=0.07 0.13
Physical Activity				x^2=0.57 0.13	x^2=0.18 0.12	x^2=0.20 0.12	x^2=0.02 0.17	x^2=0.02 0.15	x^2=0.21 0.12	x^2=0.49 0.09	x^2=0.37 0.10
NSAID Use[c]					x^2=0.36 0.08	x^2=0.51 0.11	x^2=0.25 0.11	x^2=0.001 0.18	x^2=0.04 0.14	x^2=0.008 0.12	x^2=0.04 0.14
Fruit and Vegetable Servings						x^2=0.96 0.05	x^2=0.02 0.17	x^2=0.49 0.09	x^2=0.44 0.09	x^2=0.39 0.10	x^2=0.19 0.12
BMI							x^2=0.0005 0.23	x^2=0.49 0.07	x^2=0.63 0.05	x^2=0.27 0.11	x^2=0.75 0.07
Sex								x^2=0.52 0.06	x^2=0.50 0.03	x^2=0.02 0.17	x^2=0.01 0.19
Age									x^2=0.69 0.04	x^2=0.11 0.13	x^2=0.28 0.12

Ethnicity										x^2=0.80 0.06	x^2=0.27 0.11
Folate											x^2<0.0001 0.28
B12											

+ Positive relationship
- Negative relationship
. Unclear relationship/no relationship
BMI-Body Mass Index
NSAID-Non-steroidal anti-inflammatory
MET-Metabolic equivalent task
[a]All final variables selected for inclusion were categorical. Values below x^2 report Cramer' V correlation statistics.
[b]Categories defined separately for men and women by drinking recommendations from Canadian Drinking Guidelines.
[c]Either prescription or non-prescription use.

Table B-0-3 Interaction analysis by adenoma case on the relationship between exposures of interest and LINE-1 DNA methylation for *MTHFR*

Lifestyle Exposure[a]	*MTHFR* (CC) Interaction p-value[d]	*MTHFR* (CT/TT) Interaction p-value[d]
Alcohol Consumption[b]	0.52	0.86
Smoking	0.86	0.12
BMI	0.61	0.63
NSAID Use[c]	0.37	0.87
Physical Activity	0.54	0.58
Fruit and Vegetable Consumption	0.61	0.05

[a]Exposures represent categorical versions of each variable
[b]Categories defined separately for men and women by drinking recommendations from Canadian Guidelines on Substance Abuse and Addiction.
[c]Either prescription or non-prescription use in the past year
[d]Interaction p-value extracted from models adjusted for age and sex, and mutually adjusted for all other exposures of interest.

Table B-0-4 Interaction analysis by adenoma case on the relationship between exposures of interest and LINE-1 DNA methylation for *MTR*

Lifestyle Exposure[a]	*MTR* (AA) Interaction p-value[d]	*MTR* (AG/GG) Interaction p-value[d]
Alcohol Consumption[b]	0.48	0.80
Smoking	0.65	0.12
BMI	0.06	0.34
NSAID Use[c]	0.16	0.73
Physical Activity	0.25	0.33
Fruit and Vegetable Consumption	0.68	0.04

[a]Exposures represent categorical versions of each variable
[b]Categories defined separately for men and women by drinking recommendations from Canadian Guidelines on Substance Abuse and Addiction.
[c]Either prescription or non-prescription use in the past year
[d]Interaction p-value extracted from models adjusted for age and sex, and mutually adjusted for all other exposures of interest.

Table B-0-5 Interaction Analysis by adenoma case on the relationship between exposures of interest and LINE-1 DNA methylation for *MTRR*

Lifestyle Exposure[a]	***MTRR* (AA) Interaction p-value[d]**	***MTRR* (AG/GG) Interaction p-value[d]**
Alcohol Consumption[b]	0.82	0.79
Smoking	0.90	0.30
BMI	0.45	0.69
NSAID Use[c]	0.92	0.36
Physical Activity	0.25	0.35
Fruit and Vegetable Consumption	0.67	0.24

[a]Exposures represent categorical versions of each variable
[b]Categories defined separately for men and women by drinking recommendations from Canadian Guidelines on Substance Abuse and Addiction.
[c]Either prescription or non-prescription use in the past year
[d]Interaction p-value extracted from models adjusted for age and sex, and mutually adjusted for all other exposures of interest