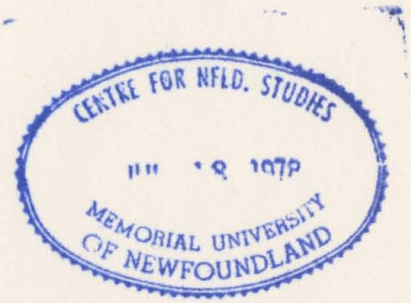
THE CLINICAL AND MOLECULAR EPIDEMIOLOGY OF INHERITED COLORECTAL CANCER IN NEWFOUNDLAND AND LABRADOR

TYLER WISH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The Clinical and Molecular Epidemiology of Inherited Colorectal Cancer in Newfoundland & Labrador

By

Tyler Wish

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Abstract

Background

Colorectal cancer (CRC) is a hereditary disease and approximately one-third of all patients have a family history of CRC. A greater understanding of the clinical and molecular features associated with hereditary CRC may lead to improved screening and patient outcomes. The purpose of this thesis is to investigate the clinical, molecular and environmental features that are associated with CRC patients who have a family history of the disease.

Methods

Incident population-based CRC patients from Newfoundland and Labrador were prospectively identified from the Newfoundland Colorectal Cancer Registry (NFCCR). Eligible index patients (n = 1,173) were diagnosed at less than 75 years of age and eligible study controls (n = 1,603) were identified through random digit dialing. Consenting patients (n = 750) provided a blood sample and permission to access medical records and tissue blocks. Biological specimens underwent molecular testing for germline mutations in the mismatch repair genes (i.e. Lynch Syndrome), tumour microsatellite-instability and for the somatic *p.V600E BRAF* mutation. Patients and controls completed family history, personal history and food frequency questionnaires.

Results

Thirty-two percent of index patients (n = 179 / 553) had at least one first-degree relative (FDR) affected by CRC. High-risk patients contained either a pathogenic mismatch repair gene variant (n = 17), or satisfied high-risk family history criteria, defined by either the familial CRC type X (FCCTX) (n = 15) or modified-FCCTX criteria (n = 16). The risk of CRC in family members of patients identified as FCCTX and modified-FCCTX is similar, but is significantly less when compared to Lynch syndrome. Twenty-six percent of non high-risk patients had at least one FDR affected by CRC. Patients who had either a synchronous or metachronous tumour or a *V600E BRAF* mutation tumour were associated with a significantly greater family history of CRC compared to patients without either of these features. Patients who have a *V600E BRAF* mutation tumour are significantly associated with diabetes, smoking and inversely associated with non-steroidal anti-inflammatory drugs.

Conclusions

The incidence of hereditary colorectal cancer in NL is high. A small subgroup of patients satisfies high-risk criteria, but the etiology of the disease is unknown for the majority of these families and requires further investigation. The *V600E BRAF* mutation and the occurrence of multiple tumours are associated with an elevated family history of CRC and may be useful markers of increased risk for screening purposes. Metabolic and inflammatory mechanisms may be important factors in the etiology of patients who have a *BRAF* mutation tumour.

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Role of the Author

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Abbreviations

AC-1	Amsterdam I Criteria
AFAP	Attenuated familial adenomatous polyposis
BER	Base excision repair
BMI	Body mass index
CI	Confidence interval
CIN	Chromosomal instability
CIMP	CpG island methylator phenotype
CRC	Colorectal cancer
FAP	Familial adenomatous polyposis
FCCTX	Familial colorectal cancer syndrome type X
FHQ	Family history questionnaire
GWA	Genome wide association
HNPCC	Hereditary non-polyposis colon cancer
HR	Hazard ratio
HRT	Hormone replacement therapy
HPP	Hyperplastic polyposis
ICG-HNPCC	The International collaborative group on Hereditary Non-Polyposis Colorectal Cancer
IHC	Immunohistochemistry
IGF	Insulin-like growth factor
LS	Lynch syndrome
LR%	Lifetime risk percent
MAP	*MUTYH*-associated polyposis
MAPK	Mitogen-activated protein kinase
MLPA	Multiplex ligation-dependent probe amplification
MGMT	O-6-methylguanine-DNA methyltransferase
M-FCCTX	Modified-FCCTX
MMR	Mismatch repair
MP	Mixed polyps
MSI-H	Microsatellite-instability high
MSS	Microsatellite-stable
MT	Multiple tumour
NFCCR	Newfoundland Colorectal Cancer Registry
NP - CRN	Nonpolypoid colorectal neoplasm
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
PHQ	Personal history questionnaire
RCT	Randomized controlled trial
RR	Relative risk
SIR	Standardized Incident Ratio
SNP	Single nucleotide polymorphism
SSA	Sessile serrated adenoma
TSA	Traditional serrated adenoma
USPSTF	U.S. Preventive Services Task Force
Wt	Wild type

Manuscripts

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4. Wish T, Green R, Younghusband HB, et al. Nonsyndromic familial microsatellite-stable colorectal cancer in the population: Incidence, cancer-risk, and molecular pathology. *Preparing to submit.*

5. Warden G, Harnett D, Younghusband HB, Wish T, et al. Familial colorectal cancer in Newfoundland: Lessons in gene discovery. *In preparation.*

6. Wish T, Wang P, Woods MO, et al. Diabetes, NSAIDs, smoking and the development of *V600E BRAF* colorectal cancer. *In preparation.*

7. Hyde AJ, Wish T, Fontaine D, et al. Unsupervised hierarchal cluster analysis of colorectal cancer. *In preparation.*

Chapter 1 – Introduction

1.1 Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer and third leading cause of cancer-related mortality in the United States and Canada. The lifetime probability of developing colorectal cancer in Canada is estimated to be 7.1% for males and 6.3% for females [1, 2]. Age-standardized incident rates across Canada for the period 1994-2005 have been stable [2] and mortality rates continue to decline (1.3% per year for males and 1.7% per year for females) as a result of earlier detection and improvements in treatment. However, age-standardized incidence rates in the Province of Newfoundland and Labrador (NL) are increasing. Furthermore, the Province has the highest incidence of colorectal cancer in the country and colorectal cancer mortality rates in NL are approximately twice those in British Columbia. These geographical disparities are not fully understood, but may be related to differences in environment, familial and genetic risk factors, and possibly related to differences in colorectal cancer screening participation rates. It is clear, however, that the disease represents a major public health burden for the Province and that further research is necessary.

The molecular etiology of inherited colorectal cancer is poorly understood. Although numerous genetic risk factors have been linked to colorectal cancer susceptibility, they explain little of the disease risk [3]. For a small proportion of colorectal cancer patients (<5%), who are affected by a known colorectal cancer syndrome (e.g. Lynch syndrome), the specific etiology is understood. However,

the etiology remains to be poorly understood for the 20% - 30% of colorectal cancer patients who appear to be affected by an inherited predisposition [3]. A greater understanding of the etiology of inherited colorectal cancer will translate into better preventative strategies, improved patient outcomes and enhanced utilization of health care resources.

Colorectal cancer is a complex and heterogenous disease. In the 1980s Vogelstein proposed a model of carcinogenesis that suggested that the development of colorectal cancer was driven by the progressive and stepwise accumulation of specific genetic alterations. The progressive accumulation of genetic and epigenetic alterations and the erosion of the genome's integrity (i.e. genomic deletions, insertions, mutations and rearrangements) is believed to a principal driver of colorectal carcinogenesis, and is referred to as genomic-instability. Furthermore, the molecular etiology of colorectal carcinogenesis is now recognized as heterogenous and results from at least three different molecular mechanisms, which are also referred to as pathways of carcinogenesis [4].

Many of the recent advancements in our understanding of colorectal carcinogenesis have arisen from the emerging field of molecular pathology epidemiology [5]. This field of study takes a transdisciplinary approach and has numerous advantages over traditional epidemiological methods, particularly when investigating complex and heterogenous diseases such as colorectal cancer. For this reason, the present study has taken a molecular pathology approach to the investigation of colorectal cancer by simultaneously considering traditional

epidemiological risk factors (e.g. smoking) in conjunction with clinical and family history information, and molecular markers of carcinogenesis.

Participants for this research project were recruited from Newfoundland, which is a valuable resource for the investigation of complex diseases [6]. It is also an ideal region to investigate the hereditary basis of colorectal cancer because families have tended to be large over several generations, family members have settled near the ancestral community, little in-or-out migration has occurred since the initial settlements in the late eighteenth and early-nineteenth centuries [6] and also because Newfoundland has the highest incidence of inherited colorectal cancer in the world [7]. The Newfoundland population is also attractive from a molecular genetics perspective, as the population has the greatest generalizibility to Caucasian populations when compared to twelve other founder populations [8].

1.2 Research Purpose

The purpose of this thesis is to generate insight into the etiology of hereditary colorectal cancer, which can then be utilized to improve the effectiveness of colorectal cancer screening. The objectives are to investigate the:

1. Clinical and molecular epidemiology of colorectal cancer
2. Risk of developing colorectal cancer in first-degree family members according to family history criteria
3. Patient and patient-tumour characteristics that are associated with colorectal cancer patients who have a family history of disease
4. Dietary and lifestyle factors associated with colorectal cancer patients according to tumour-molecular phenotype

Chapter 2 – Literature Review

2.1 Colorectal Cancer Etiology

2.1.1 Inherited Colorectal Cancer

Well-defined inherited colorectal cancer (CRC) syndromes (e.g. Lynch syndrome and polyposis syndromes) account for approximately 5% of all incident colorectal cancer patients [3]. Lynch syndrome is the most prevalent inherited colorectal cancer syndrome and is characterized by an elevated susceptibility to develop gastrointestinal, gynecological, brain, skin and other malignancies [9]. It is an autosomal dominant condition caused by inactivating germline mutations of genes, *MLH1*, *MSH2*, *MSH6* and *PMS2*, which code for proteins that perform DNA mismatch repair (MMR). The two most prevalent polyposis syndromes are familial adenomatous polyposis (FAP) and attenuated familial adenomatous polyposis (AFAP), both of which are caused by inactivating mutations of the *adenomatous polyposis coli* (*APC*) gene [10]. Although the number of polyps can range considerably for these two syndromes, patients affected by either FAP or AFAP typically present with numerous adenomatous polyps of the colon. Prophylactic management for these patients is necessary, as the lifetime risk of developing colorectal cancer is nearly one hundred percent for patients inheriting an APC mutation. *MUTYH*-associated polyposis (MAP) is also associated with the development of adenomatous polyps, but is caused by mutations of the *MUTYH* gene, and is inherited as an autosomal recessive condition.

The rare hamartomatous polyposis syndromes are another subgroup of inherited colorectal cancer syndromes. These syndromes, which include Peutz-

Jeghers syndrome and Juvenile polyposis syndrome, are caused by mutations of *STK11*, *SMAD4*, and *BMPR1A [10].* There are also rare syndromes of unknown etiology that are associated with the development of numerous hyperplastic polyps. The best example is known as hyperplastic polyposis syndrome (HPS). These syndromes will be discussed in further detail.

Based on the findings from kindred and twin studies [11-13], it is estimated that 20% — 30% of all colorectal cancer cases are directly attributable to inherited factors. The relative contribution of genetic and environmental risk factors is unclear, but inherited factors appear to play a prominent role [13, 14]. Unfortunately, however, the specific etiology for these patients is poorly understood. Newfoundland may have the highest incidence of inherited colorectal cancer in the world. Green *et al* reported [7] that Newfoundland has a significantly greater incidence of patients with at least one affected first-degree relative (FDR) when compared to thirteen other population-based studies conducted worldwide. Furthermore, it was subsequently demonstrated [15] that the high incidence of inherited colorectal cancer in Newfoundland is not entirely attributable to founder mutations in known susceptibility genes, which suggests that the high incidence of inherited colorectal cancer in the province may be caused by novel susceptibility factors. However, the high incidence of hereditary colorectal cancer in Newfoundland may also be related to increased environmental risk, founder effects in unknown genes, or a better ascertainment of family risk [7, 15].

2.1.3 Genetic Susceptibility

2.1.3.1 Low-Penetrant Susceptibility Loci

Accumulating evidence indicates that the etiology of colorectal cancer is complex and heterogenous. A recent analysis [16] reported that as many as 170 genetic variants may be implicated with colorectal cancer susceptibility. Genome-wide association studies (GWAs) have discovered multiple low-penetrant loci that are associated with colorectal susceptibility. To date, colorectal cancer susceptibility is significantly associated with loci located at: 8q24 [17, 18], 8q23.3 [19], 10p14 [19], 11q23 [20], 15q13 [21], and 18q21 [21, 22]. A more recent meta-analysis [23] has identified four additional risk loci at: 14q22.2, 16q22.1, 19q13.1, and 20p12.3. These findings are consistent with the common disease-common variant hypothesis [24], which hypothesizes that common variants are likely to underlie much of the disease susceptibility for common diseases. Unfortunately, however, the cancer risk associated with any single variant is small and collectively these risk factors account for only a small percentage of the excess familial risk — approximately 6% [23]. However, the risk conferred by these loci is likely to be a conservative estimate, since the identified loci are often not the causative alleles, but loci that that are in linkage disequilibrium with the causative variant.

It is unlikely that additional common (minor allele frequency > 30%) low-risk variants will be discovered, as GWA studies have been designed to have sufficient statistical power to identify these variants. It is probable, however, that less common susceptibility alleles exist, some of which likely have greater penetrance. It may be possible to identify these alleles with different

technological and analytical approaches, such as exome or whole-genome sequencing.

The mechanisms through which the identified risk alleles modify colorectal cancer susceptibility are largely unknown; particularly as many of the risk loci identified thus far are positioned at regions of the genome that lack protein-coding transcripts. It has been hypothesized that some variants may promote cancer predisposition by influencing gene expression at distant sites. In support of this hypothesis, two recent studies [25, 26] have provided evidence demonstrating how a particular variant (rs6983267), which resides in a region lacking protein-coding transcripts, promotes colorectal carcinogenesis. One study [25] suggests that the risk-associated variant at 8q24 (G-allele) physically interacts with the *MYC* promoter, located 335kb away, to promote colorectal carcinogenesis by increasing expression of *MYC*. Another study [26] proposes that the risk variant causes genomic rearrangements around 8q24 and results in a copy number increase for a transcription-factor binding site that promotes colorectal carcinogenesis by over-activation of the Wnt signaling pathway. While the definitive answer is unclear, these studies have illuminated mechanisms through which non protein-coding variants may affect gene expression and promote carcinogenesis.

Genetic studies using high-risk families and affected sib-pairs are strategies that have also identified loci associated with colorectal cancer susceptibility [27-32]. For example, a susceptibility locus has been identified at a region on chromosome 9q [27] and later confirmed by additional studies [28, 29]. A genome wide sib-pair analysis of 70 affected families [32] identified susceptibility

loci at 3q [30, 31] and at 7q31.31. The causative genes have yet to be identified. Nevertheless, the results support the hypothesis that multiple susceptibility alleles are implicated in colorectal cancer predisposition.

2.1.3.2 Low-Penetrant Genetic Variants

Low-penetrant gene variants are also linked with colorectal cancer susceptibility [33]. An example of a low-penetrant genetic variant is the *I1307K* variant of the *APC* gene, which is associated with a 2 – fold increase in colorectal cancer risk and is present in approximately 6% of the Ashkenazi Jews [34]. The *TGFBR1*6A* variant is another example, and it confers a 1.2-fold increase in colorectal cancer risk which, although modest, may account for 3% of the total colorectal cancer burden [35]. Furthermore, the *TGFBR1*6A* risk allele is more prevalent in high-risk families [36] and may be implicated in FCCTX [37]. It has recently been demonstrated [38] that heterozygous *MUTYH* mutation carriers are at a slightly increased risk for developing colorectal cancer (adjusted OR, 1.48; 95% CI, 1.02 – 2.16), and *MUTYH* is now recognized as a low-penetrant risk allele.

Gene variants have also been identified as modifiers of cancer risk. The findings of two studies [39, 40] have suggested that a *p53* variant has a significant effect on the age of cancer onset in Lynch syndrome patients, but a third study [41] found no such association. Other variants, such as *HRAS1*VNTR* and *MTHFR*677V*, are suspected of influencing colorectal cancer risk, but their mechanism of action is unknown and further studies are required [35].

2.2 Colorectal Cancer Risk Factors

2.2.1 Family History

A family history of colorectal cancer is a significant risk factor for developing the disease [12, 42-45]. A meta-analysis [43] of 27 case-control and cohort studies indicated that the relative risk (RR) for developing colorectal cancer was 2.25 (95% Confidence Interval (CI), 2.00 – 2.53) if a first-degree relative was affected. The risk is significantly greater if more than one first-degree relative is affected (RR, 4.2; 95% CI, 3.01 – 6.08). Furthermore, the risk is greatest if a first-degree relative was affected before age 45 years (RR, 3.87; 95% CI, 2.40 – 6.22) and reduces if diagnoses was between 45 – 49 years (RR, 2.25; 95% CI, 1.85 – 2.72) or greater than 59 years age (RR, 1.82; 95% CI, 1.47 – 2.72). Having a first-degree relative affected with an adenoma is also a significant risk factor (RR, 1.99; 95% CI, 1.55 – 2.55), as is having a second-degree relative affected by colorectal cancer (RR, 1.73; 95% CI, 1.02 – 2.94) [44]. It has been reported [44, 45] that the risk of colorectal cancer is greater if a sibling is affected (RR, 2.79; 95% CI, 2.36 – 3.29) rather than if a parent is affected (RR, 2.07; 95% CI, 1.83 – 2.34). Furthermore, it has also been reported [45] that the risk of colorectal cancer is greatest in siblings of those with right-sided tumours.

2.2.2 Environmental Risk Factors

In addition to genetic factors, numerous non-heritable factors are also associated with the risk of colorectal cancer. These include: dietary factors, the determinants and consequences of insulin resistance syndrome and smoking.

2.2.2.1 Dietary Factors

Dietary factors have been implicated as colorectal cancer risk factors not only because of the physical interaction with the digestive tract, but also because of the association between the western-diet and a high incidence of colorectal cancer. Although numerous dietary risk factors have been reported the findings have often been conflicting. For example, a diet rich in fruits and vegetables has been hypothesized to reduce colorectal cancer risk but, in an analysis of 31 case-control studies, only seventeen found an association between the disease and low consumption of fruit and vegetables [46]. The consumption of meat products is also associated with colorectal cancer risk. It is thought that meat cooked at high temperatures may contain carcinogenic heterocyclic amines, polycyclic aromatic hydrocarbons, and N-nitroso compounds [47]. However, two meta-analyses [48, 49] investigating meat consumption have found non-significant and inconsistent results. Similarly, no clear association has been found between a high-fat diet and colorectal cancer [50]. A high-fiber diet was suggested to reduce colorectal cancer risk in a large European cohort study [51], but two additional cohort studies [52, 53] found no clear association. Conversely, alcohol consumption has consistently been linked with a slight increase in the risk of colorectal cancer. In a pooled analysis of 8 cohort studies [54], alcohol use was associated with a significantly elevated risk of colorectal cancer (RR, 1.23; 95% CI 1.07 – 1.42).

2.2.2.2 Lifestyle Factors

Insulin resistance syndrome (IRS) – also known as metabolic syndrome – is caused by a defect in insulin action (insulin resistance) and a compensatory increase in insulin secretion (hyperinsulinemia) [55]. It is associated with a cluster of metabolic abnormalities, including: obesity (particularly visceral adiposity), impaired glucose tolerance, hypertriglyceridemia, hypertension, type 2 diabetes, and atherosclerotic cardiovascular disease. According to the National Cholesterol Education Program [56], a diagnosis of metabolic syndrome requires three or more of the following: Hypertension, central adiposity (waist circumference), BMI greater than 27 kg/m^2, low HDL cholesterol, hypertriglyceridemia, impaired glucose tolerance. Meeting the definition for metabolic syndrome has consistently been associated with a 50% increase in the risk of colorectal cancer [57-60]. Additionally, many of the individual determinants and consequences of insulin resistance are independently linked with colorectal cancer [61-63]. For example, excess central adipose tissue is associated with increased colorectal cancer risk via its adverse effects on insulin sensitivity, inflammation, growth factors and steroid hormones [64, 65]. A recent meta-analysis [66] of 31 studies found that obesity (BMI $\geq 30kg/m^2$) has a direct and independent relationship with colorectal cancer. The pooled results suggest that compared to those with a BMI of less than $25kg/m^2$, obese individuals have an increased risk of colorectal cancer (RR, 1.19: 95% CI, 1.11 - 1.29). However, obesity appears to present a greater risk in men (RR, 1.41; 95% CI, 1.30 – 1.54) than in women (RR, 1,08; 95% CI, 0.98 – 1.18). The reasons for this disparity are not fully understood, but it has been hypothesized that the protective effect of

exogenous estrogen in postmenopausal women may counterbalance the negative impact of excess adipose tissue [65]. Alternatively, it has been suggested that body mass index is a poorer indicator of visceral adipose tissue in women relative to men [67].

Physical inactivity is also a risk factor that is independent of obesity and other potential confounding factors. The results of a meta-analysis [68] of 40 case-control and cohort studies suggested that a significant reduction in colorectal cancer risk could be achieved with physical activity. Furthermore, any amount of activity appears to be beneficial [69] and the risk decreases with increasing activity [70].

Hypertension, elevated blood glucose, hyperinsulinemia and type 2 diabetes are metabolic abnormalities associated with insulin resistance syndrome that are also independently associated with colorectal cancer [61]. Type 2 diabetes is a consequence of long-term insulin resistance and is associated with a significant increase in the risk of developing colorectal cancer. The findings of a recent meta-analysis [63] suggest that type 2 diabetes increases the risk of colorectal cancer by thirty percent (95% CI, 20% - 40%).

The underlying mechanisms linking insulin resistance syndrome with colorectal cancer are not fully understood, but the available evidence suggests that hyperinsulinemia is the likeliest cause. This is supported by animal model and *in vitro* studies, which have demonstrated that insulin strongly promotes carcinogenesis via its effects on cellular proliferation and apoptosis [61, 65].

2.2.2.3 Smoking

Epidemiological evidence [71-74] utilizing cross-sectional, case-control and prospective cohort study designs strongly indicates that smoking is a significant colorectal cancer risk factor. For example, two recent cross-sectional studies [72, 73] found current smokers to be significantly more likely to develop advanced neoplasia than non-smokers. A case-control study [71] found that 40 year+ smokers were 1.92 (95% CI, 1.13 – 3.28) times more likely to develop colorectal cancer than non-smokers. A prospective cohort study [74], which followed 25,279 middle-aged Japanese men for 7 years, found that past smokers (RR, 1.73; 95% CI 1.04 – 2.87) and current smokers (RR, 1.47; 95% CI 0.93 – 2.34) were significantly more likely to develop colorectal cancer when compared to never smokers. Additionally, it was reported that a greater number of cigarettes smoked per day and an earlier age of smoking onset increase the risk of colorectal cancer.

The mechanism by which smoking increases the risk of colorectal cancer is not entirely clear. Tobacco smoke contains likely carcinogens including polycyclic hydrocarbons, aromatic amines and benzene [75]. More recently, smoking has been linked to increased DNA methyltransferase activity, suggesting that it may promote carcinogenesis via epigenetic mechanisms [76, 77]. The consistency of the evidence suggests that smoking increases colorectal cancer risk. However, several questions remain unanswered, such as: how does smoking modify colorectal cancer risk in certain subgroups that have a genetic predisposition?

2.3 CRC Prevention

A number of therapies are thought to function as colorectal cancer chemopreventatives agents. The list includes: non-steroidal anti-inflammatory drugs (NSAIDs), COX-2 inhibitors, dietary supplements, hormone replacement therapy (HRT) and statins.

The chemopreventative attributes of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have received the most scrutiny and are the most promising. Aspirin and NSAIDs function to inhibit inflammatory pathways that play an important role in colorectal carcinogenesis [78]. The US Preventive Services Task force (USPSTF) recently published two systematic reviews [79, 80] examining the benefits and harms of aspirin and non-aspirin NSAIDs for the prevention of colorectal neoplasia. Their analysis found that the regular use of aspirin reduced the incidence of adenomas in RCTs (HR, 0.82; 95% CI 0.7 – 0.95), cohort studies (HR, 0.72; 95% CI 0.61 – 0.85) and in case-control studies (HR, 0.87; 95% CI, 0.77 – 0.98). Furthermore, the efficacy of aspirin was increased when used at high doses and when used for periods longer than ten years. Similarly, the use of non-aspirin NSAIDs was associated with a reduced risk of developing colorectal cancer in cohort (HR, 0.61; 95% CI 0.48 – 0.77) and case-control studies (HR, 0.70; 95% CI 0.63 – 0.78). The caveat is that the use of high dose aspirin and non-aspirin NSAIDs were also found to be associated with dose-related increases in the incidence of gastrointestinal complications. Based on the available evidence, the USPSTF's recommendation was to not support the regular use of aspirin or NSAIDs for the prevention of colorectal cancer, arguing that the benefits do not outweigh the potential harms [79, 80].

It has been hypothesized that folate supplementation may reduce the risk of colorectal cancer. However, this has been refuted by a recent study [81] in which 1,021 patients with a history of colorectal adenomas were randomized to receive either 1 mg folic acid per day or placebo, and separately randomized to receive low-dose aspirin, high-dose aspirin, or placebo. At the end of the follow-up period, those receiving folic acid were more likely to have three or more adenomas.

Calcium supplementation has also been linked with a reduced risk of colorectal cancer. However, in a pooled analysis of 10 cohort studies [82], patients in the highest quartile of total calcium intake were less likely to develop colorectal cancer (Pooled HR, 0.78; 95% CI 0.69 – 0.88) when compared to the lowest quartile. However, results from the Women's Health Initiative trial [83], a randomized double-blind placebo controlled trial, found no difference in the incidence of colorectal cancer between those who did or did not receive combined calcium-vitamin D supplementation (HR, 1.08; 95% CI 0.86 – 1.34).

From the same study cohort [84] the use of hormone replacement therapy in postmenopausal women was observed to significantly reduce the risk of colorectal cancer (HR, 0.63; 95% CI 0.43 – 0.92), which was confirmed in another case-control study [85]. However, hormone replacement therapy was subsequently linked [84] to increased breast cancer and cardiovascular disease risk, and therefore could not be recommended as a chemopreventative agent.

The risk of colorectal cancer was reduced by 47% in those who used statins for greater than 5 years, according to one case-control study [86]. However, subsequent studies [87, 88] did not confirm those initial findings and a recent

meta-analysis [89] found no benefit of statins in either randomized controlled or cohort studies.

Although some therapies appear to reduce the risk of CRC, they are unfortunately either associated with considerable toxicities or have failed to demonstrate effectiveness in RCTs. Thus, no therapy is currently recommended as a chemopreventative agent. The discovery of chemopreventative agents is challenging and is complicated by the heterogenous etiology of colorectal cancer, the long latent period of colorectal carcinogenesis and potential toxicities. However, despite these challenges it is encouraging that the development of precancerous lesions and adenocarcinoma appears to be susceptible to modification by chemopreventative agents.

2.4 Molecular Pathways of Colorectal Carcinogenesis

Approximately 30% of human genes encode for proteins that function to repair and maintain the genome [90]. The integrity of the human genome is critical for human health and dysfunction of these proteins often has severe health consequences. For example, inactivating mutations in genes that perform DNA mismatch repair (Lynch syndrome), base-excision repair (*MUTYH*-associated polyposis), double-strand break repair (*BRCA1* and *BRCA2*), and nucleotide excision repair (Xeroderma pigmentosum) result in severe cancer predisposition. Colorectal carcinogenesis progresses via a sequential accumulation of genetic and epigenetic alterations and many of these alterations affect genes that function to maintain the integrity of the genome. These observations have led to the hypothesis that genomic instability plays a crucial

role in the etiology of colorectal carcinogenesis. Unfortunately, the specific mechanisms causing genomic instability are unclear, but it is associated with the genome incurring point mutations, small deletions and insertions, and gross chromosomal alterations. Over the past decade it has become evident that genomic instability can be achieved by at least three broadly distinct molecular pathways of carcinogenesis [4]. These pathways are recognized as the: chromosomal-instability (CIN), microsatellite-instability (MSI) and serrated pathways. Although they are not entirely mutually exclusive, these pathways are relatively unique from both a molecular and clinical perspective. The molecular pathology of colorectal carcinogenesis is likely far more complex than three molecular pathways, but our current understanding of these pathways provides a framework from which to direct further research. These three pathways of carcinogenesis, along with their associated syndromes, are described in further detail below.

2.4.1 Chromosomal Instability (CIN) Pathway

The most common pathway through which colorectal cancer occurs is the chromosomal-instability (CIN) pathway. The CIN pathway is characterized by aneuploidy, numerous base substitutions, deletions, insertions, chromosomal rearrangements and copy number changes [91] and are commonly associated with mutations affecting *APC*, *KRAS*, *p53*, *PIK3CA*, and *SMAD* [92]. The *adenomatous polyposis coli* (*APC*) gene, which is part of the Wnt signaling pathway, is thought to play a principal role in the CIN pathway of colorectal carcinogenesis for several reasons: i) germline mutations of *APC* lead to severe

polyposis syndromes (e.g. FAP) [93]; ii) somatic *APC* mutations are frequently observed in sporadic colorectal tumours (70%); and iii) alterations of *APC* can be found in the earliest CRC precursor lesions and often precede all other molecular alterations [94, 95].

The *APC* gene product has a host of cellular functions, one of which is the regulation of the Wnt signaling pathway. β-catenin activates the Wnt signaling pathway by binding to transcription factors that initiate transcription of genes that promote proliferation, such as *MYC* and *CYCLIN D1*. The APC protein normally functions to negatively regulate the Wnt signaling pathway by binding β-catenin and mediating its degradation. However, mutations of *APC* often lead to truncated proteins that have impaired function, thereby allowing β-catenin to translocate to the nucleus and causing over-activation of the Wnt signaling pathway.

Components of the mitogen-activated protein kinase (MAPK) signaling pathway are also often perturbed in CIN tumours. *KRAS* is a component of the MAPK signaling pathway and it is normally deactivated by GTP hydrolysis, but specific mutations allow *KRAS* to remain in an activated state. Activation of the MAPK signaling pathway promotes increased cellular proliferation and survival. Mutations of *KRAS* are found in early adenomas, but are more frequently observed in advanced adenomas (50%) and with adenomas larger than 1 cm in diameter (58%) [96], which suggests that *KRAS* mutations occur early in tumourigenesis and promote adenoma growth, but are not necessary for malignant transformation [97].

The *p53* gene is a tumour suppressor gene that is involved in maintaining genomic stability via control of the cell cycle. In response to genotoxic stress, p53 up-regulates a number of genes that directly block the cell cycle and initiate apoptosis. Inactivating mutations of *p53* are commonly observed in tumours arising from the CIN pathway, and they confer an advantage to the cell by allowing it to evade apoptosis.

Although the above-described genes are frequently mutated in CIN tumours, they do not appear to be the primary cause of CIN. The mechanisms that cause CIN are largely unknown, but perturbations in genes that regulate the cell cycle, mitotic spindle checkpoint, centrosome number and telomeres are thought to be likely candidates. Aneuploid tumours often display additionally abnormalities during mitosis - such as abnormal centrosome number, multipolar spindles and lagging chromosomes - which strongly implicate genes that regulate the mitotic spindle checkpoint [98]. For example, mutations in *MAD* and *BUB* have been linked to CIN colorectal tumours because mutations in these genes often cause abnormalities during mitosis [99]. Abnormal centrosome number and function have also been implicated in CIN tumours. Several studies are in support of this, for example a locus at 20q13 was observed to be frequently amplified in human epithelial tumours [100] and later studies identified the *STK15* gene at that locus. Subsequently, *STK15* amplification was commonly observed in breast [101] and colon cancer cell lines [102]. Over-expression of *STK15* has been identified as a potential cause of mitotic spindle assembly abnormalities [98]. In addition to *STK15*, a number of DNA checkpoint genes are also linked to CIN, which include mutations and amplifications of *ATM*, *ATR*,

BRCA1, *BRCA2*, *PLK1* and *CDC4*. However, it remains unclear whether these alterations are causative or just permissive of CIN.

2.4.1.1 Familial Adenomatous Polyposis (FAP)

Familial adenomatous polyposis (FAP) is an autosomal dominant hereditary polyposis syndrome caused by mutations of the *adenomatous polyposis coli* (*APC*) gene. The genetic etiology of FAP was initially located to a region at 5q21 – 22 [103] and the *APC* gene was subsequently identified in clinically identified families [104]. The majority of mutations affecting *APC* occur in exon 15 and commonly result in truncated proteins. The APC protein normally binds β-catenin and mediates its degradation, but the truncated APC protein poorly binds β-catenin. This enables β-catenin to translocate to the nucleus and cause over-expression of the Wnt signaling pathway.

In classical FAP, germline mutations of *APC* are nearly 100% penetrant [105], but FAP accounts for less than 1% of all colorectal cancer. Patients typically present with hundreds to thousands of adenomatous polyps of the bowel at an early age and develop colon cancer by the fourth decade of life [106]. For this reason, patients undergo prophylactic surgery to remove the bowel. FAP is also associated with an increased risk of peri-ampullary adenoma and carcinoma, medulloblastoma, papillary carcinoma of the thyroid, hepatoblastoma, carcinoma of the stomach and congenital hypertrophy of retinal pigmented epithelium [107].

Germline mutations of *APC* are also the etiological basis for attenuated familial adenomatous polyposis (AFAP). Compared to FAP, AFAP is

characterized by fewer polyps and a tendency for proximal colonic adenomas [108].

2.4.2 Microsatellite-Instability (MSI) Pathway

Microsatellites are short repeating DNA sequences that are located throughout the genome. They are susceptible to errors during DNA replication, particularly if the human DNA mismatch repair (MMR) system is impaired. The microsatellite-instability phenotype is defined by a variation in the length of sequences in DNA derived from the tumour. The phenomenon was initially observed in the malignant colon tissue of cancer syndrome patients [109, 110] and subsequently linked to Lynch syndrome. However, 10% — 15% of non-Lynch syndrome colorectal patients (i.e. sporadic colorectal cancer patients) also have tumours that are microsatellite-unstable, suggesting that the MSI pathway has an alternative etiology. Further investigation led to the discovery that the MSI pathway in 'sporadic' colorectal cancer tumours was the result of aberrant epigenetic inactivation of *MLH1* by promoter methylation [111]. This phenomenon is strongly associated with the serrated pathway, which will be discussed separately.

The MSI pathway is characterized by a distinct clinicopathological profile. MSI tumours are often located in the proximal colon, and tend to be mucinous and poorly differentiated [112, 113]. Patients who have a MSI tumour are associated with a better prognosis than patients who have a microsatellite-stable tumour [114].

2.4.2.1 Lynch syndrome

Lynch syndrome is the most prevalent known inherited colorectal cancer syndrome and is attributable for 2% — 4% of the colorectal cancer burden [115]. Lynch syndrome caused by inactivating mutations of *MLH1, MSH2, MSH6* and *PMS2*. The corresponding proteins function to perform DNA mismatch repair and dysfunction of this system significantly elevates the risk of developing early-age tumours of the colorectal, endometrium, ovary, stomach, small bowel, hepatobiliary tract, pancreas, ureter, renal pelvis, brain and skin [116].

2.4.2.1.1 History

An American pathologist, Aldred Warthin, first documented the clinical manifestations of Lynch syndrome in a 1913 publication, which reported his findings of a cancer predisposition family "Family G". Decades later, Henry Lynch extensively researched the family history of a family known as Family N, which displayed many of the features of what is now known to be Lynch syndrome. At the time it was recognized that Family N members did not present with multiple polyps, which were known to be associated with FAP – the only known hereditary cancer syndrome at the time. Lynch continued his research efforts by updating the work founded by Warthins' Family G and by collaborating with others who had identified a similar cancer family (Family M) [117, 118]. In 1971, Lynch *et al* reported on the clinical features that identified "cancer family syndrome" which included, increased adenocarcinoma of the colon and endometrium, multiple primary malignancies, early age onset and autosomal dominant inheritance [119]. It was not until the 1990's that the

etiology of Lynch syndrome was discovered [120, 121]. During this period, The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC) was formed, which collaborated on research efforts, promoted awareness and developed clinical criteria to identify Lynch syndrome families [122]. As a result of the technological advancements made in molecular genetics, a vast amount of knowledge has been acquired regarding hereditary colorectal cancer, which will be discussed below.

2.4.2.1.2 Etiology

The human DNA MMR system functions specifically to repair single-base mismatch errors and insertion-deletion loops that occur during replication and recombination. DNA MMR function was originally identified in E.coli, and homologues of the E.coli MMR genes have been found in yeast and mammals [123]. The link between mismatch repair function and Lynch syndrome was first identified in 1993 when a susceptibility locus was mapped and assigned to chromosome 2p by linkage analysis [120]. A human homologue of the *MSH2* gene in yeast [124] was cloned [125] and was subsequently linked to Lynch syndrome based on germline mutations in several families with severe cancer predisposition [126]. Likewise, *MLH1* was cloned by its yeast homologue [127, 128] and germline mutations were linked to Lynch syndrome in 1994 [128]. Later, mutations in two additional genes implicated with human DNA MMR were found to cause Lynch syndrome - *PMS2* [129], followed by *MSH6* [130, 131].

The human DNA MMR system is comprised of numerous proteins that collectively function to perform DNA mismatch repair. However, MLH1, MSH2,

MSH6, and PMS2 are critical for proper functioning of the MMR system. These proteins, along with others, form heterodimeric complexes that play specific roles identifying and repairing DNA mismatch errors. The MutS complex, which couples MSH2 with MSH6 or MSH3, functions to identify mismatch errors and binds to DNA by exchanging ADP for ATP. The MutL complex involves MLH1 coupling with PMS2, PMS1, or MLH3, which binds to MutS dimers that have recognized DNA errors. The MutS-MutL complex subsequently activates endonuclease activity to repair mismatch errors. This model implies that some degree of redundancy exists, which is also observed clinically. Since *MLH1* and *MSH2* are critical for proper mismatch repair function, they account for a majority of known MMR mutations. In this model MLH1 can form heterodimers with either MLH3 or PMS1 and therefore a mutation of *MLH3* or *PMS1* appears to have little or no phenotypic effect [132].

Germline mutations in MMR genes can result in proteins with loss of interaction domains or with changes in conformation that ultimately impair their ability to interact and function. Mutation carriers have one normal allele that is thought to be sufficient for MMR function, and tumourigenesis occurs only after inactivation of the wild-type allele, which can occur by either loss of heterozygosity, somatic point mutation or promoter methylation. Impaired MMR function undermines genomic integrity by significantly increasing the mutation rate and leads to numerous DNA errors occurring throughout the genome in a non-random fashion. Segments of the genome that contain microsatellite repeat sequences are most susceptible to mismatch errors. Consequently, MMR-deficiency often leads to inactivation of many tumour

suppressor genes that contain microsatellite-like repeats, such as *TGFBR2* [133], *IGFIIR* [134], *PTEN* [135], as well as genes involved in Wnt signaling [136] and apoptotic pathways [137]. The *TGFBR2* gene product controls a number of signaling pathways involved in growth inhibition and cellular death. Inactivating mutations of *TGFBR2* are commonly observed and cause the receptor to be insensitive to the growth inhibitory effects of TGF-B1. Furthermore, activating mutations of B-catenin are frequent and cause over-expression of the Wnt signaling pathway by up-regulating the transcription factors *MYC* and *CYCLIN D1*. Inactivating mutations of *BAX*, a gene that is critical for cell death, are common and enable tumour cells to evade apoptosis.

In 1994 the international Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC) established a database of MMR mutations. In 2003, the ICG-HNPCC and the Leeds Castle Polyposis Group (LCPG) merged to form INSIGHT (International Society for Gastrointestinal Hereditary Tumours). At that time, 448 pathogenic mutations were documented in 748 affected families from around the globe. To date, germline mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2* are definitively associated with Lynch syndrome. However, mutations of several other MMR genes such as, *MLH3*, *PMS1* and *MSH3* are less likely to be pathogenic, but their involvement is not yet fully understood [138]. Mutations in *MLH1* and *MSH2* account for the majority of Lynch syndrome cases (approximately 90%), and mutations of *MSH6* and *PMS2* account for the remainder. Most mutations are frameshift and nonsense mutations that lead to truncated proteins, but missense mutations are also reported in more than one-third of all mutations involving *MLH1* and *MSH6* [138].

Founder mutations are pathogenic mutations that account for a large fraction of the total disease burden in a specific population. Founder mutations occur by a single mutation carrier introducing a novel mutation into a population, which over a period of generations may be responsible for a disproportionate amount of disease. For example, a specific *MSH2* mutation (c.942+3A →T) is estimated to account for 5% — 10% of all Lynch syndrome patients worldwide [139]. Certain features of a population increase the probability of a founder mutation flourishing, such as: isolation, rapid population growth and chance [35]. Two founder mutations of *MLH1* have been identified in the Finnish population [140, 141] and a single *MLH1* mutation affects the Swiss population [142]. *MSH2* founder mutations have been identified in North American [143], Ashkenazi Jew [144] and Newfoundland populations [145]. A Newfoundland family (Family C) was integral to the original linkage study that identified the susceptibility locus on 2p [120]. This mutation was subsequently identified in 12 independently ascertained Newfoundland families, and was suggestive of a founder mutation by a common haplotype of markers [145].

2.4.2.1.3 Diagnosis and Testing

The ICG-HNPCC first established clinical criteria to identify Lynch syndrome patients in 1991 [122]. Fulfilling the Amsterdam I criteria (AC-I) requires [122] that: a) at least three relatives should have histologically verified colorectal cancer and one of them should be a first-degree relative of the other two; b) two successive generations should be affected; c) one of the affected must be diagnosed before fifty years of age, and d) FAP must be excluded. In 1998, the

Amsterdam II criteria (AC-II) were introduced [116], which expanded the original AC-I criteria to include cancers of the endometrium, ureter, renal pelvis and small bowel. The Amsterdam criteria were criticized for lacking clinical sensitivity and subsequently, the Bethesda guidelines [146], followed by the revised Bethesda guidelines in 2004 [147], were created in order to identify patients who should undergo MSI and or IHC testing.

A workshop for the European guidelines for the clinical management of Lynch syndrome in 2007 assessed the performance of the Amsterdam and revised Bethesda criteria. It determined that the sensitivity of the Amsterdam and Bethesda criteria for the detection of mutation carriers was 40% and 90%, respectively [148]. The positive predictive value of the Amsterdam and revised Bethesda criteria is approximately 50% and 10% — 20%, respectively [149]. The workshop concluded that the revised Bethesda criteria were appropriate for the selection of patients whose tumours should undergo molecular testing for microsatellite-instability, considering the high-cost associated with alternatively testing all patient tumours [148].

Microsatellite-instability testing involves comparing matched normal and malignant tissue by using a panel of 5 to 10 specific microsatellite markers. Various thresholds for instability are used, but generally microsatellite instability (MSI-H) is determined if greater than 30% of markers demonstrate instability; MSI-Low if 10 – 30% are instable; and MSS if <10% of markers are instable [150]. Approximately 90% of colorectal tumours that arise from Lynch syndrome exhibit MSI [121]. However, MSI is also observed in approximately 15% of sporadic colorectal tumours as a result of epigenetic silencing of *MLH1* [111]. As

a result, the specificity of MSI testing for the detection of MMR mutation carriers is not ideal, but MSI testing is a reliable, sensitive and clinically useful test for identifying potential Lynch syndrome patients. Patients with a MSI-H tumour are recommended to undergo immunohistochemistry testing (IHC) testing and or screening for known germline mutations.

2.4.2.1.4 Lynch Syndrome Prediction Algorithms

A diagnosis of Lynch syndrome is seldom straightforward and it often involves a combination of personal and family history information, as well as molecular diagnostics. The most effective and cost-efficient strategy is still debated and several groups have developed MMR-mutation prediction algorithms in an effort to improve diagnostic efficiency. These prediction models include the Lieden model [149], $PREMM_{1,2}$ [151], MMRpredict [152] and MMRpro [153]. The various prediction models typically utilize personal history and family history information to estimate the likelihood of being a MMR mutation carrier. In validation cohorts the $PREMM_{1,2}$, MMRpredict and MMRpro prediction models have reported area under the curve (AUC) values of 0.80 (95% CI, 0.76 – 0.84), 0.82 (95% CI, 0.72 – 0.91) and 0.83 (95% CI, 0.78 – 0.88), respectively. Interestingly, despite utilizing different information and having different algorithms, the three models have reported similar AUC values in validation cohorts.

The clinical utility of these models for use in the general population has been questioned, particularly since these models were developed and validated using high-risk patient cohorts. Two studies [154, 155] have independently assessed

the clinical utility of these prediction models. The first study [154] evaluated these models using a cohort of 72 referred high-risk patients and reported that the PREMM, MMRpro, MMRpredict and Lieden models, but not the Myraid genetics model, performed better than the Amsterdam II criteria. The reported AUC values were 0.75 (95% CI, 0.64 – 0.87), 0.86 (95% CI, 0.76 – 0.96), 0.90 (95% CI, 0.82 – 0.97), 0.90 (95% CI, 0.81 – 0.98) and 0.93 (95% CI, 0.86 – 0.99), for the Myriad, MMRpredict, Lieden, MMRpro and $PREMM_{1,2}$ models, respectively. The authors concluded that the models performed well. However, the study cohort was a high-risk group of referred patients. A more appropriate study by Green *et al* [155] evaluated the performance of these models using a large cohort of population-based patients. Green *et al* reported that the predication algorithms performed reasonably well (AUC values ranged from 0.91 – 0.96) and outperformed the revised Bethesda criteria. However, they tended to overestimate the probability of low-risk patients having a MMR mutation. After correcting for family size, the best performing model was MMRpredict, which achieved a sensitivity of 94% (95% CI, 73 – 99%) and a specificity of 91% (95% CI, 88 – 93%).

2.4.2.1.5 Cancer Risk

Lynch syndrome patients have a substantially increased liability to develop colorectal and extracolonic malignancies. Quantifying the cancer risk associated with Lynch syndrome is an important facet of providing evidence-based health care, as accurate estimates of cancer risk enables strategies to be implemented that reduce cancer-related mortality. However, obtaining accurate and precise

estimates of cancer risk can be challenging since the findings can be severely biased by the design and analysis of such studies. For example, soon after the etiological basis of Lynch syndrome was discovered, several authors [156-159] reported erroneously high lifetime estimates for developing colorectal cancer (as high as 82%), largely because of using severely affected and clinically ascertained families. Subsequent studies [160-162] utilized more appropriate study designs, patient cohorts and statistical methods to minimize the potential for bias, and these studies have found lower estimates of cancer risk. For example, a study [163] of *MLH1* and *MSH2* mutation carriers, which used a population-based cohort as opposed to clinically ascertained families and incorporated both affected and unaffected mutation-positive family members, found a later age of colorectal cancer onset and a reduced penetrance (69% in men and 52% in women) compared to earlier studies. That being said, the findings of a recent study [164] has estimated that the lifetime colorectal cancer risk for men (cumulative risk, 66.1%; 95% CI, 59.5% – 76.2%) and women (cumulative risk, 42.7%; 95% CI, 36.6% – 52.8%) Lynch syndrome patients to be quite high.

Lynch syndrome patients are also at increased risk for developing extracolonic malignancies of the stomach, small bowel, renal pelvis, ureter, ovaries, biliary tract and brain. Results of a large study [165] of *MLH1* and *MSH2* mutation carriers indicated that the greatest extracolonic risk were cancers affecting the urological tract (cumulative incidence, 8.4%; 95% CI, 6.6% - 10.8%). The lifetime risk of ovarian (cumulative incidence, 6.7%; 95% CI, 5.4% - 9.1%), gastric (cumulative incidence, 5.8%; 95% CI, 4.4% - 7.7%), small bowel (cumulative incidence, 4.3%; 95% CI, 3.1% - 5.9%), brain (cumulative incidence,

2.1%; 95% CI, 1.5% - 2.9%), and biliary-pancreatic cancer (cumulative incidence, 4.1%; 95% CI, 2.8% - 5.9%) was also reported. The authors suggested that cancers of the urologic tract and ovaries occur frequently enough in some Lynch syndrome families to justify cancer screening.

There is also evidence for mutation-specific differences in cancer risk. For example, a study [164] that examined the incidence of cancer in 147 kindred affected by *MLH1*, *MSH2* and *MSH6* mutations discovered that male *MLH1* mutation carriers (RR, 342; 95% CI, 264 – 442) were at significantly greater risk for developing colorectal cancer than male *MSH2* mutation carriers (RR, 78; 95% CI, 57 – 107). Female *MLH1* carriers were also at greater risk of CRC (RR, 76; 95% CI, 58 – 103) than female *MSH2* carriers (RR, 46; 95% CI, 33 – 65), but this result was not statistically significant. The risk of developing endometrial cancer in *MLH1* (RR, 31; 95% CI, 20 – 50), *MSH2* (RR, 47; 95% CI, 35 – 64) and *MSH6* (RR, 18; 95% CI, 6 – 55) mutation carriers appears to be similar. There is also evidence [165] to indicate that the incidence of urological and ovarian cancers is significantly greater in *MSH2* mutation carriers than in *MLH1* carriers.

Furthermore, even within the same MMR gene, different mutations appear to influence phenotype. For example, a study [166] of three Newfoundland families, who had different mutations affecting *MSH2*, found significant differences between families for the risk of developing extracolonic malignancies. However, the risk of developing colorectal cancer was comparable.

MSH6 mutations account for only a minority (approximately 7%) of known MMR gene mutations causing Lynch syndrome [138]. There is evidence [167-170] to suggest that relative to *MLH1* and *MSH2* mutations, *MSH6* mutations

have a lower penetrance, and that patients affected by *MSH6* mutations have a delayed age of onset of cancer, but a higher risk of developing endometrial cancer. A study [171] of two Swedish founder *MSH6* mutations found the lifetime risk for developing any Lynch syndrome-associated cancer to be 89% in women and 69% in men. Despite having a later-age onset of cancer, the penetrance of *MSH6* mutations appears to be high, suggesting that intensive counseling, management and surveillance is necessary, but that early-age screening may not be as critical as with *MLH1* and *MSH2* mutation carriers.

PMS2 mutations account for a small proportion of Lynch syndrome patients, but they are likely underreported as molecular detection has proved difficult [9]. As a consequence, cancer risk in patients affected by *PMS2* mutations is unclear. However, detection methods are improving, which has enabled better ascertainment of patients with *PMS2* mutations. The findings of a recent study [172], which ascertained a large cohort of colorectal cancer patients whose tumours stained negative for *PMS2* on IHC, demonstrated that *PMS2* mutations are common in these patients (>60% were mutation positive). The mean age of colorectal cancer onset in 55 monoallelic *PMS2* mutation carriers was 50 years, with a range of 23 – 77 years. Interestingly, *PMS2* mutation carriers rarely had a family history that fulfilled the Amsterdam criteria (9%), however 65.5% satisfied the revised Bethesda criteria, while the remaining (25.5%) failed to satisfy any family history risk criteria. The risk of colorectal cancer was comparable in males (lifetime risk, 20%; range, 11% - 34%) and females (lifetime risk, 15%; range, 6% - 35%). There was some indication that carriers were at increased risk of extracolonic tumours associated with Lynch syndrome, but the finding was not

statistically significant ($P = 0.3$). The lifetime risk of any Lynch syndrome-associated malignancy in males (cumulative risk, 25%; range, 16% - 48%) and females (cumulative risk, 32%; range, 21% - 53%) is less than what has been typically reported for *MLH1* and *MSH2* mutation carriers.

The clinical heterogeneity that is observed amongst Lynch syndrome patients is suggestive of yet to be identified modifier genes. This has been supported by a study of Lynch syndrome patients [173], which found that the risk of colorectal cancer was decreased by variants located at 8q24.3 and 11q23.1. Further research in this field will allow for even greater precision in estimates of cancer risk.

The evidence suggests that males are at greater risk for developing colorectal cancer than females, and that *MLH1* mutation carriers are at greater risk than *MSH2* mutation carriers. However, the risk of endometrial cancer is comparable for *MLH1* and *MSH2* carriers. *MSH2* mutation carriers have a greater propensity to develop extracolonic cancers of the urological tract and ovaries. The phenotype in *MSH6* mutation carriers is variable, but the penetrance appears to be high despite reports of later-age onset of cancer. *PMS2* mutation carriers have reduced penetrance with a lifetime risk of 25% – 32% for any Lynch syndrome-associated cancer. Larger studies are needed to refine estimates so that mutation, gender and tumour-specific screening protocols can be put into clinical practice with confidence.

2.4.2.1.6 Accelerated Carcinogenesis

The malignant transformation of an adenomatous polyp to invasive carcinoma is estimated to take 10 – 15 years in average-risk population - defined as not having either a family history of colorectal cancer or a predisposing condition. In patients with Lynch syndrome, the rate of malignant transformation is accelerated and may take as little as 2 – 3 years [106, 112, 174]. However, Lynch syndrome patients who develop colorectal cancer appear to have a better prognosis compared to non-Lynch syndrome colorectal cancer patients who have microsatellite-stable tumours [175, 176].

2.4.2.1.7 Cancer Screening and Surveillance

A 15-year prospective study [177] of Lynch syndrome patients found that three-yearly colonoscopies resulted in a 62% reduction in colorectal cancer incidence, as well as a significant reduction in colorectal cancer-mortality. A reduction in colorectal cancer-mortality as a result of screening and surveillance has also been found by two other studies [178, 179]. A more recent study [180] by Stuckless *et al* evaluated the effectiveness of colonoscopic screening in 322 *MSH2* mutation carriers from Newfoundland and found that screening was associated with a decreased risk of colorectal cancer, a later age of onset and better survival.

The benefits of screening and surveillance for reducing cancer incidence and mortality in Lynch syndrome patients are clearly evident. The consensus regarding optimal surveillance for Lynch syndrome patients is two-yearly colonoscopy initiated between the ages of 20 – 25 years and continued to age 80

years if the patient is in good health. For families that are mutation-negative but display familial clustering of colorectal cancer, colonoscopy is recommended every 3 – 5 years, beginning 5 – 10 years before the earliest age of colorectal cancer diagnosis in the immediate family [148]. Female Lynch syndrome patients have a high lifetime risk of developing endometrial cancer (39.4%; 95% CI, 30.8% – 46.9%) [164] and for this reason are recommended to have annual endometrial screening beginning at age 30 – 35 years, although the effectiveness of this screening has yet to be demonstrated. Prophylactic hysterectomy may also be considered, as one study [181] demonstrated a significant reduction in endometrial and ovarian cancer in Lynch syndrome patients who underwent hysterectomy compared to those who did not. Similarly, despite that the evidence for efficacy is minimal, it is recommended that those with a family history of cancer affecting the ureter, renal pelvis, stomach or small bowel seek appropriate screening [9].

2.4.3 Serrated Neoplasia Pathway of Carcinogenesis

It is evident that an alternative pathway of colorectal carcinogenesis co-exists with the CIN and MSI pathways of carcinogenesis. Recognized as the serrated neoplasia pathway [182], it is associated with serrated adenomatous precursor lesions, which are differentiated from conventional adenomatous polyps by molecular and morphological features. The serrated pathway may be implicated in 20% of colorectal cancers and particularly with interval cancers [183], i.e. colorectal tumours arising within a short period of colonic screening. The etiology of the serrated pathway is unknown, but activating mutations of the

MAPK pathway and dysfunction of gene promoter methylation appears to play a prominent role. Furthermore, susceptibility to develop colorectal cancer via the serrated pathway appears to be influenced by both genetic and environmental factors [184, 185].

2.4.3.1 Serrated Precursor Lesions

In the late 1980s investigators reported that a rare syndrome associated with the development of multiple colonic hyperplastic polyps, now recognized as hyperplastic polyposis syndrome (HPS), was associated with an elevated risk of developing colorectal cancer [186]. Further investigations revealed that colonic lesions occurring in patients who had HPS were morphologically distinct from the innocuous hyperplastic polyp [187]. In 2005, a classification system for the heterogenous group of lesions referred to as serrated polyps was proposed [188], which recognized several different types of serrated polyps. The hyperplastic polyp is the most prevalent type and accounts for 80% - 90% of all serrated lesions. These polyps are typically small (i.e. less than 5mm in dimension), have little malignant potential and are commonly found in the distal colon of the elderly. The defining morphological feature of the hyperplastic polyp is the appearance of serration along the upper half of the crypt. The advanced serrated polyps include: traditional serrated adenomas (TSAs), sessile serrated adenomas (SSAs) and mixed polyps (MP). Unlike the hyperplastic polyp, these lesions have considerable malignant potential, can be large (> 1cm) and are distributed throughout the entire colon. Traditional serrated adenomas are characterized by a hybrid of dysplasic (typical of an adenomatous polyp) and serrated architecture

[189]. TSAs tend to occur in the distal colon and are frequently associated with mutations of either *BRAF* or *KRAS*, as well as with methylation of the O-6-methylguanine DNA methyltransferase (*MGMT*) gene. One study [190] indicated that 15.9% of all polyps were serrated adenomas, but estimates of their prevalence ranges considerably. The sessile serrated adenoma was first described in 1996 by Torlakovic *et al* [191] and these lesions are characterized by architectural features such as, T or L-shaped crypts. Unlike hyperplastic polyps where serration is limited to the upper half of the crypt, SSAs are defined by serration that extends to the base of crypts.

2.4.3.2 Molecular Features

The serrated pathway of carcinogenesis is strongly associated with two molecular abnormalities: the CpG island methylator phenotype (CIMP) and the somatic *V600E BRAF* mutation. The cause of these aberrations is unclear, but it is suspected that they are implicated with the etiology of the serrated pathway.

2.4.3.2.1 CpG Island Methylator Phenotype (CIMP)

CpG dinucleotides are dinucleotides located throughout the human genome. Certain regions of the genome contain higher frequencies of CpG dinucleotides and are known as CpG islands. CpG islands are located at the 5′ region of approximately 50% of all genes and are typically in the unmethylated state [192]. Epigenetic methylation of CpG islands can impair DNA transcription by either directly inhibiting transcription factors from binding to promoter regions, or by inducing changes in chromatin structure. Epigenetic modification

of CpG islands has numerous important functions in human biology [193], but in some circumstances the regulation of DNA methylation becomes deregulated and consequently pathogenic. As an example, aberrant DNA methylation promotes carcinogenesis by inactivating tumour-suppressor genes [194-199].

Approximately 20% – 30% of colorectal tumours display extensive methylation of CpG islands and this phenomenon has been suggested to represent a distinct subtype of colorectal cancer, termed CpG island methylator phenotype (CIMP) [194, 199]. The etiology of CIMP is unknown and whether this phenomenon signifies a distinct molecular subtype of colorectal cancer has been debated [200]. However, accumulating evidence suggests that CIMP is a unique subtype of cancer that is strongly associated with the serrated pathway of carcinogenesis. It has been demonstrated that aberrant DNA methylation is pathogenic, affects a large number of known tumour suppressor genes and is observed in aberrant crypt foci and early adenomas, which suggests that aberrant methylation occurs early in colorectal carcinogenesis [111, 201-203].

Tumours displaying CIMP can be categorized as high (CIMP-H) and CIMP-Low (CIMP-L) [204, 205]. CIMP-H tumours display extensive methylation of CpG islands and tend to be associated with microsatellite-instability (MSI-H), the somatic *V600E BRAF* mutation and sessile serrated adenomas. Conversely, CIMP-L tumours display a lower level of aberrant DNA methylation and are more strongly associated with the distal colon, with lesions exhibiting low-level microsatellite-instability (MSI-L) or microsatellite-stability, methylation of *O-6-methylguanine methyltransferase* (*MGMT*) and mutations of *KRAS* rather than

BRAF [206]. Furthermore, the CIMP-L phenotype appears to be more strongly associated with traditional serrated adenomatous precursor lesions.

2.4.3.2.2 V600E BRAF Mutation

BRAF is a component of the Ras/Raf/MEK/MAPK signal transduction pathway, which plays an important role in cellular growth, proliferation, and apoptosis [207]. The somatic V600E *BRAF* mutation is a T-to-A transversion that results in the constitutive activation of *BRAF*. The mutation is frequently observed in melanoma and colorectal cancer, and is recognized as a primary genetic event in carcinogenesis and as having mild oncogenic effect [208]. Approximately 10% – 18% of all colorectal tumours are estimated to have the mutation [209-211]. The mutation is strongly associated with CIMP and the MSI phenotype [199, 209-212], but it occurs rarely with MSI tumours arising from Lynch syndrome [213]. Furthermore, the mutation is seldom observed in adenomas and hyperplastic polyps, but is commonly associated with traditional serrated adenomas and sessile serrated adenomas [214-217]. For example, approximately 30% of traditional serrated adenomas and 78% – 90% of sessile serrated adenomas are reported to harbour the mutation. Furthermore, the *BRAF* mutation appears to be associated with females, smoking and proximal tumour location [214, 217, 218] and *V600E* tumours typically present with mucinous morphology, tumour-infiltrating lymphocytes and tend to be poorly differentiated [219].

2.4.3.3 Serrated Pathways of Carcinogenesis

There is accumulating evidence for alternative pathways of carcinogenesis existing within the serrated pathway. One pathway is characterized by the strong association between CIMP, *V600E BRAF*, microsatellite-instability and the sessile serrated adenoma [199, 209, 211, 212, 214-217, 220]. This pathway is referred to as the sessile serrated adenoma (SSA) pathway, or simply, the serrated pathway. Sessile serrated adenomas arise from the microvesicular hyperplastic polyp and in the SSA pathway of carcinogenesis epigenetic inactivation of specific tumour suppressor genes, *p16INK4a* and *IGFBP7*, enables *BRAF* mutant cells to evade oncogene-senescence [221-224] and allows for uncontrolled proliferation and progression of serrated lesions. Although the SSA pathway is associated with microsatellite-instability, it appears that epigenetic inactivation of *MLH1* is a late-occurring step in the malignant transformation of SSAs [225, 226]. However, not all SSA pathway neoplasia exhibits microsatellite-instability and there is a subset of tumours that are CIMP-high, *V600E BRAF* and microsatellite-stable.

The traditional serrated adenoma (TSA) pathway is characterized by an association between low-level CIMP, microsatellite-instability low (MSI-L), methylation of *MGMT*, and mutations of *KRAS* [206, 227-231]. The precursor lesions that are associated with this pathway are unclear, but it has been proposed that they may represent a hybrid or fusion between lesions associated with both the traditional adenoma-carcinoma and serrated pathways [216, 232].

2.4.3.4 Serrated Pathway Risk Factors

2.4.3.4.1 Environmental Risk Factors

Evidence suggests that the etiology of the serrated neoplasia pathway is associated with certain environmental risk factors. These include a low dietary consumption of calcium, folate, and fiber, as well as a high dietary consumption of fat, alcohol and meat [233-237]. Factors associated with insulin resistance syndrome, such as obesity and smoking, are also linked with the serrated pathway [236-248]. There is some evidence [236] to suggest that risk factors for right-sided versus left-sided serrated polyps may be different, which is likely due to the heterogenous nature of serrated polyps.

Smoking is the strongest environmental risk factor associated with the serrated pathway. A substantial body of evidence [249] links smoking with the risk of developing colorectal cancer. Recently, however, the association between smoking and colorectal cancer has been recognized to be greatest for those with hyperplastic polyps [242, 243] and for patients whose tumours display microsatellite-instability [244, 245], CIMP and the *V600E BRAF* mutation [237, 245-248]. The mechanisms by which smoking influences the development of serrated pathway colorectal cancer is poorly understood, but it may be related to a link recently found [76, 77] between smoking and increased CpG island methylation. It may also be related to a finding [250, 251] that cigarette smoke activates the aromatic hydrocarbon receptor, which can lead to methylation of *p16* and *p53*.

2.4.3.4.2 Genetic Risk Factors

A genetic predisposition to develop colorectal cancer via the serrated pathway has been suggested [184]. Evidence for an inherited predisposition to develop colorectal cancer via the serrated pathway is linked to two colorectal cancer predisposition syndromes, namely hyperplastic polyposis syndrome and serrated pathway syndrome [185]. The genetic etiology of HPS is unknown, but it is clinically identified by having: a) at least five histologically diagnosed hyperplastic polyps proximal to the sigmoid colon, two of which are greater than 10mm in diameter; or b) any number of hyperplastic polyps occurring proximal to the sigmoid colon in an individual who has a FDR with hyperplastic polyposis; or c) more than 30 hyperplastic polyps of any size but distributed throughout the colon [252]. HPS is a rare condition and is typically diagnosed relatively late in life (50 – 70 years of age). A family history of HPS has been reported in some families, which suggests a possible genetic predisposition [253]. The data is insufficient to establish the risk of developing colorectal cancer in those with HPS, however the risk is thought to be greatest in patients with large, atypical and dysplastic polyps. Furthermore, it is suggested that the precursor lesions associated with HPS harbor the capacity to undergo rapid malignant transformation. One report [186] described three HPS patients who developed colorectal cancer despite 2-yearly colonoscopy. Another report [254] has suggested that 3-yearly colonoscopy surveillance was insufficient for some families with serrated polyps.

Young *et al* [255] described families who were affected by colorectal cancer in a manner consistent with autosomal dominant inheritance. Furthermore, family

members exhibited a predisposition to develop sessile serrated adenomas and adenocarcinomas that had clinicopathological features consistent with the sessile serrated adenoma pathway – proximally located, MSI-variable, *V600E BRAF* tumours. These observations suggest that the development of serrated pathway colorectal cancer may be caused by penetrant inherited factors in some patients. Young *et al* referred to this strong predisposition to develop serrated pathway colorectal cancer as serrated pathway syndrome (SPS).

Further evidence for a genetic predisposition to develop colorectal cancer via the serrated pathway comes from two recent studies [211, 214], which discovered an elevated family history of colorectal cancer associated with patients who have sessile serrated adenomas or tumours with the *V600E* mutation, both of which are strongly associated with the sessile serrated adenoma pathway. In an unselected series of patients undergoing colonoscopy, patients with sessile serrated adenomas, compared to patients with other colonic lesions, were more likely to have a family history of colorectal cancer (42% versus 25%; $P > 0.05$), and a greater polyp burden ($P < 0.001$) [214]. The second study [211], a large unselected population-based study of colorectal cancer patients, found that amongst patients with MSS tumours, patients who had a *V600E BRAF* tumour were significantly more likely to have a family history of colorectal cancer (OR, 4.2; 95% CI, 1.65 – 10.84). However, the association with family history was not observed for patients who had MSI-H tumours.

2.4.4 MUTYH-Associated Polyposis (MAP)

The base-excision repair (BER) system is a 'care-taker' mechanism that functions to detect and repair DNA damage caused by oxidative damage [256]. Although numerous proteins are involved in BER the MUTYH protein is critical for the detection and removal of adenine residues that incorrectly pair with 8-oxo-7,8-dihydro2'deoxyguanosine. Failure to repair this mispairing results in distinctive G:C to T:A transversions at the next round of DNA replication. Germline *MUTYH* mutations were discovered in patients who clinically presented like FAP, but had an autosomal recessive pattern of inheritance, and did not harbor pathogenic mutations of the *APC* gene. Further examination revealed characteristic somatic G:C to T:A mutations in the tumours of these patients, which implicated the BER system and *MUTYH* gene specifically. To date, more than 80 mutations of *MUTYH* have been catalogued, but two specific mutations (Y165C and G382D) account for approximately 80% of all mutations reported in Caucasian populations [257, 258].

MUTYH-associated polyposis (MAP) is a recessively inherited condition that presents similarly to FAP and AFAP. However, it is highly variable and diagnosis is typically at a later age than FAP. No other defining features of MAP have been identified, but some extra-colonic manifestations have been observed [259]. The findings of a recent study [260] of 276 MAP patients, suggested that the syndrome is also associated with an elevated risk of duodenal, ovarian, bladder and skin cancers. Several population-based case-control studies [38, 261, 262] have estimated that 0.4% - 1.0% of colorectal cancer patients are carriers of homozygous or compound heterozygous *MUTYH* mutations. Homozygous and

compound heterozygous mutation carriers appear to be at substantial risk for developing colorectal cancer. A recent study [38] found an adjusted odds ratio of 18.1 (95% CI, 2.5 – 132.7) for the odds of developing colorectal cancer. However, the cancer risk associated with heterozygous *MUTYH* mutations has been a subject of debate. Several studies [261, 263, 264] have linked heterozygous mutation carriers with a non-significant increase in colorectal cancer risk, but a recent large case-control study [38], which screened for a larger number of *MUTYH* mutations than previous studies, reported that heterozygous carriers were at increased risk of developing colorectal cancer (Adjusted OR, 1.48; 95% CI, 1.02 – 2.16). The cancer risk associated with a heterozygous *MUTYH* mutation carrier is now widely acknowledged as a low-penetrant risk allele.

2.4.5 Familial Colorectal Cancer Type X (FCCTX)

In 1991, the ICG-HNPCC established clinical criteria (Amsterdam I Criteria) to identify high-risk colorectal cancer families for gene identification purposes. In order to fulfill the Amsterdam I criteria a family needed to satisfy all criteria: a) three cases of histologically verified colorectal cancer in two generations, with one affected being a FDR of the other two, and b) one patient diagnosed with colorectal cancer before the age of 50 years, and c) Familial adenomatous polyposis must be ruled out. The clinical criteria are still used to identify potential Lynch syndrome patients, but once the molecular etiology of Lynch syndrome was discovered, it was recognized that not all families fulfilling Amsterdam I criteria were affected by MMR gene mutations.

A seminal paper [37] by Lindor *et al* investigated colorectal cancer patients who fulfilled the AC-1 in order to investigate the clinicopathological and cancer risk differences between patients with and without evidence of tumour MMR-deficiency. The principle finding was that patients without MMR-deficiency had a significantly reduced familial incidence of colorectal cancer (SIR, 2.3; 95% CI, 1.7 – 3.0) compared to those with MMR-deficiency (SIR, 6.1; 95% CI, 5.2 – 7.2). Additionally, age at colorectal cancer diagnosis was found to be significantly later in families of patients without MMR-deficiency (61 versus 49 years) and there was little evidence for developing extracolonic malignancies. The authors concluded that AC-1 patients without evidence of mismatch repair dysfunction likely represented a genetically heterogenous group of inherited colorectal cancer syndromes that are etiologically and clinically different from Lynch syndrome. The authors identified patients fulfilling the AC1 without evidence of MMR-deficiency as familial colorectal cancer type X (FCCTX).

Subsequent studies have reported similar findings as Lindor *et al* [37]. For example, one study [265] compared *MLH1* and *MSH2* mutation carriers to colorectal patients who had a family history satisfying the AC-1, but who had tumours without evidence of MMR-deficiency. That study also found that FCCTX families had a later age of onset of colorectal cancer than mutation carriers (Median 41 vs. 55 years; $P < 0.001$). Additionally, FCCTX patients were found to be more likely to have left-sided tumours (68% versus 14%; $P < 0.01$) and less likely to have synchronous or metachronous tumour s($P < 0.017$) or have extracolonic tumours ($P < 0.001$). Notably, the ratio of adenoma to carcinoma was higher in those with normal MMR function ($P < 0.03$), suggesting that the

transformation from adenoma to carcinoma was reduced in FCCTX patients compared to those with MMR-deficiency. The findings of a large prospective study [266] conducted in Spain, suggested that 60% of patients with a family history fulfilling either the Amsterdam I or II criteria had a microsatellite-stable tumour. Similar to previous findings, patients without evidence of MMR-deficiency were older at diagnosis ($P = 0.6$) and more likely to have distally located tumours ($P = 0.15$). The family members of these patients were less likely to be affected by colorectal cancer ($P = 0.011$) and had a later age of diagnosis ($P = 0.036$). The authors of one other study [267] have also reported that a large proportion of Amsterdam I criteria colorectal cancer patients have a MMR-proficient tumour (40%). These patients were found to be significantly older at diagnosis compared to patients who had a MMR-deficient tumour (53 versus 41 years; $P < 0.001$) and more frequently had left-sided tumours ($P = 0.001$). Additionally, family members were less likely to have a synchronous or metachronous tumour ($P < 0.001$) or to have an extracolonic tumour ($P = 0.001$).

It is speculated that the etiology of FCCTX could be explained by a highly penetrant risk variant, multiple low-penetrant risk alleles, shared lifestyle factors or even statistical chance. However, the accumulating evidence suggests that FCCTX has numerous etiologies. This hypothesis is supported by numerous linkage studies [27-31] of FCCTX-like families, which have found linkage to multiple regions of the genome. For example, a sib-pair analysis [27] found evidence for linkage to a region on chromosome 9 (9q22.2 – 31.2), in a pattern consistent with autosomal dominant disease. Linkage to this region was confirmed by two additional studies [28, 29]. A high-density genome-wide

linkage study [30] found linkage to 3q21 – q24. A genome-wide linkage analysis [31] of 30 Swedish families who had dominant colorectal cancer family histories, found no association with the previously described region at 9q22. However, that study did find strong evidence for linkage to 3q21.1 – q26.2. Most recently, a comprehensive genome-wide linkage analysis of seven FCCTX-like families, performed by Middeldrop *et al* [268], also found evidence for linkage to 3q, but overall the findings were inconclusive. Collectively, these findings support the hypothesis that FCCTX is likely a heterogenous disease.

2.5 Colorectal Cancer Screening

Treatment of colorectal cancer is highly successful if diagnosed in the early stages. Five-year survival is approximately 90% for localized disease, but decreases to 68% for regional disease (Lymph node involvement) and 10% if distant metastasis has occurred [269]. These probabilities highlight the reality that screening and early detection are critical for positive patient outcomes. The natural history of colorectal cancer includes a long preclinical phase (10 – 15 years) and detectable precursor lesions, which provide opportunities for screening and intervention. There is convincing evidence from several randomized controlled trials [270-274] that screening and subsequent intervention (polypectomy) reduces both the incidence of colorectal cancer and colorectal cancer related mortality. For example, a study [274] recently demonstrated that a screening program that used repeated annual or biennial guaiac fecal occult blood tests (FOBTs) and endoscopic follow-up of positive tests, reduced colorectal cancer mortality by sixteen percent (95% CI, 10% – 22%)

after 12 – 18 years. Unfortunately, despite the benefits of screening, a large number of adults are non-compliant with current screening recommendations or have never received any form of screening at all [275, 276]. In 2006, among US adults aged 50 years and older, the prevalence of screening with an endoscopic procedure in the previous 10 years was just 56.3% [275]. Furthermore, screening prevalence is significantly lower in some ethnic minorities, in lower socioeconomic classes and in the uninsured [276].

In an effort to promote screening and to provide consensus evidence-based screening recommendations, several organizations have issued joint guidelines for colorectal cancer screening and surveillance, namely, a) The American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer and the American College of Radiology [277] and b) The U.S Preventive Services Task Force (USPSTF) [278].

In 2006 the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology provided a joint recommendation regarding colorectal cancer screening and surveillance. In 2008, the group updated its screening and surveillance recommendations for the early detection of colorectal neoplasia [277]. The recommendations for screening distinguished between tests that detect adenomatous polyps and tests that primarily detect cancer. For tests that detect polyps and cancer, average risk men and women should begin screening at age 50 years, with one of the following regimens: flexible sigmoidoscopy every 5 years, double-contrast barium every 5 years, computed topographic colonography every 5 years or colonoscopy every 10 years. For tests that primarily detect cancer, any of the following regimens are

recommended: annual guaiac-based fecal occult blood test with high sensitivity for cancer, annual fecal immunochemical test with high sensitivity for cancer or stool DNA test with high sensitivity for cancer (interval uncertain). Patients with a family history of colorectal cancer (colorectal neoplasia in a 1st degree relative before age 60 years, or colorectal neoplasia affecting two or more 1st degree relatives at any age) are recommended to being screening (colonoscopy every 5 years) at age 40 years or 10 years before the youngest case in the immediate family.

In 2008 the USPSTF updated its screening recommendations [278]. The USPSTF's assessment concluded that the net benefit of screening was high for average-risk persons aged between 50 – 75 years who followed either a) annual high-sensitivity fecal occult blood testing, or b) flexible sigmoidoscopy every 5 years – combined with high-sensitivity fecal occult blood testing every 3 years, or c) colonoscopy ever 10 years. The net benefits for individuals 76 – 85 were small, and the net benefit of screening does not outweigh the harm for individuals greater than 85 years of age. In addition, the USPSTF concluded that there was insufficient evidence to assess the effectiveness of fecal DNA tests, and as well, concluded that there was insufficient evidence to assess the net benefit and harm of CT colonography.

Colorectal cancer surveillance protocols are applicable to patients found to have a polyp during screening. Polyps are a significant risk factor for colorectal cancer and therefore decreased screening intervals are recommended for patients found to have polyps. Surveillance guidelines have been developed to identify patients that are at high-risk of neoplasia recurrence from those who are low-risk,

and are based on studies that have characterized polyp features predictive of future recurrence. For example, a study [279] of 3,121 asymptomatic veterans, aged 50 – 75 years, that were screened and had repeat examinations approximately 5.5 years later, found that patients who had multiple tubular adenomas, a large tubular adenoma (> 1 cm), villous histology or an adenoma with high-grade dysplasia at baseline, were significantly more likely to develop advanced neoplasia.

Further to its recommendations for screening, the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology jointly issued a statement regarding surveillance [277]. Patients are recommended to have repeat screening colonoscopy at 10 years if no neoplasia is found at initial screening; Repeat screening at 5 years if 1 or 2 small (< 1cm) tubular adenomas are found; and 3-year interval colonoscopies if at increased risk for advanced lesions, defined as having an advanced lesion (> 1cm polyp, villous histology, or high-grade dysplasia) or 3 or more adenomatous polyps.

Although the guidelines were evidence-based, a recent study [280] has questioned the evidence that has formed the Task Force's surveillance recommendations. This particular study [280] evaluated the ability of the current surveillance guidelines to stratify high-risk from low-risk patients. In 1,905 patients who had an adenoma at baseline colonoscopy, the probability of advanced adenoma recurrence at 4 years was 0.09 (95% CI, 0.07 – 0.11) among patients with high-risk adenomas at baseline and 0.05 (95% CI, 0.04 – 0.06) among those with low-risk adenomas at baseline. Although this difference is statistically significant, it has been argued that the result may not be clinically relevant.

Furthermore, the study found that only the villous histology component of the surveillance guidelines was significantly predictive of advanced adenoma recurrence in a multivariate model. Adenoma size, high-grade and multiple adenomas were not independently predictive of recurrence. This result also questions the predictive value of the current surveillance guidelines to effectively discriminate between high and low risk patients.

In addition to adenomatous polyps, various other colonic lesions are being recognized as precursor lesions with malignant potential. Two types of lesions that have been increasingly implicated in carcinoma predisposition are the advanced serrated polyp and non-polypoid colorectal neoplasia (NP-CRN). The advanced serrated polyp, which includes sessile serrated adenomas, serrated adenomas and mixed polyps are now recognized to harbor malignant potential. Current opinion suggests that larger serrated polyps located in the proximal colon should be removed, but that smaller polyps located in the distal colon are less likely to undergo malignant transformation.

Non-polypoid colorectal neoplasia (NP-CRN) describes gastrointestinal lesions that are depressed or flat and only recently was their malignant potential accepted [281, 282]. Reports [283] from Asian populations in the 1980s and 90s suggested that NP-CRNs were prevalent and had malignant potential, but not until recently did a study examine the prevalence of NP-CRN in a North American population and characterize their association with colorectal cancer. In 2008, a cross-sectional study [284] of 1,819 patients undergoing colonoscopy found the proportion of NP-CRNs to be 9.4% (95% CI, 8.0% – 10.8%) of all identified colonic polyps. Importantly, NP-CRNs were found to be much more

likely to contain carcinoma than polypoid lesions (OR, 9.8; 95% CI 3.9 – 24.4). NP-CRN presents a potentially challenging diagnostic problem. It appears that these lesions are prevalent and harbor malignant potential, but they are difficult to detect with current optical screening modalities. Additionally, resection of NP-CRNs is challenging and the current evidence regarding recurrence risk is insufficient. For these reasons it has been hypothesized [283] that NP-CRNs may explain a substantial proportion of interval cancers.

Current screening practices are effective, but there are deficiencies in screening participation, infrastructure and knowledge. Although screening prevalence is increasing in the United States, it remains low. In an effort to improve screening participation, organizations have issued consensus statements that provide alternative screening strategies for patients and their physicians. It has been suggested [275] that participation in screening could improve with increased public awareness, incentives for healthcare providers to recommend screening, improvements in infrastructure to remind patients about screening and increased access to care. Funding clinical research should also be a priority, as there remains an uncertainty regarding best clinical practice, the effectiveness of emerging novel screening modalities (CTC and Stool DNA tests), the best markers to stratify high-risk from low-risk patients, and the significance of non-polypoid and serrated lesions.

Chapter 3 – Research Methods

3.1 Patients and Methods

This study prospectively identified incident colorectal cancer patients from the Newfoundland Colorectal Cancer Registry (NFCCR). Patients were eligible if diagnosed with colorectal carcinoma (ICD-9; colon 153.0 – 153.9, excluding 153.5 (appendix); Rectum 154.0 – 154.1) between January 1, 1999 and December 31, 2003, and if they were less than 75 years of age at diagnosis. From 1,173 identified eligible patients, 750 (64%) patients or their proxy consented to take part in the study. Study controls were identified through random digit dialing and were frequency matched to patients for sex and 5-year age strata. From 1,603 potentially eligible controls identified though random dialing, 44.8% (n = 717) agreed to participate in the study.

Patients were asked to provide a blood sample and to grant permission to access medical records and tissue blocks. Biological specimens provided by patients had undergone a series of molecular analyses; which included testing for tumour microsatellite-instability (MSI), immunohistochemistry (IHC), *MLH1* promoter methylation, p. *V600E BRAF* mutation (*V600E BRAF*), and testing for mutations in mismatch repair (MMR) genes. MSI status was assigned as MSI-high (MSI-H, >30% of markers tested unstable), MSI-low (MSI-L, 10%-30% of markers unstable), or microsatellite stable (MSS, <10% markers unstable); however since only a small number of tumours were identified as MSI-Low we combined them with the MSS tumours. Thus, we assigned tumours as either MSI-H (>30% of markers unstable) or MSS (<30% of markers unstable). DNA

from patients who fulfilled clinical criteria for familial adenomatous polyposis was tested for *APC* mutations; first for those mutations that were previously observed in Newfoundland; then by sequencing to identify other APC mutations. Additionally, all patients were tested for *MUTYH* mutations. Patients who had an *APC* or *MUTYH* mutation, or who satisfied clinical criteria for FAP or MAP, were excluded from this study.

Pathology was reviewed for all available tumours. One representative tumour slide from each patient's tumour had been reviewed and scored for several histological features, including Crohn's-like lymphocytic reaction and tumour-infiltrating lymphocytes (TILS), as described previously [285]. Tumour grade and histology were determined from the original pathology reports. Tumour location was obtained from the records of the Newfoundland cancer registry. Proximal location was defined as proximal to the splenic flexure. Mucinous component was defined as the presence of any mucin dissecting into stroma surrounding a tumour gland. This definition includes tumours with a mucinous histology, but also those with histologic heterogeneity, in which any area of the tumour displays dissecting mucin. The occurrence of a synchronous or metachronous tumour is referred to as having multiple tumours.

Patients and controls were asked to complete a family history questionnaire (FHQ). Information obtained from the FHQ enabled pedigrees to be constructed. The cancer history of each family member was recorded as follows: the cancer status (affected: yes or no), the type of cancer, and age at diagnosis, or the age at last follow-up or death if unaffected by cancer. Index patients and their families were excluded from the study for being non-informative if >50% of first-degree

relatives (FDRs) were missing data necessary for statistical analysis (e.g. patient age could not be calculated).

The pedigree for each index patients was assessed and patients were identified as high-, intermediate-, or low-risk according to the following family history criteria. High-risk patients were identified as those who were affected by Lynch syndrome (i.e. harbored a pathogenic mismatch repair gene variant) or who otherwise fulfilled the familial colorectal cancer type X (FCCTX) [37] or modified-FCCTX (M-FCCTX) criteria, both of which are based on the Amsterdam 1 criteria (AC-1). The FCCTX criteria [37] are as follows:

i. At least three relatives affected by CRC; with one affected relative being a FDR of the other two affected.
ii. At least two successive generations affected by CRC.
iii. At least one relative affected by CRC diagnosed before the age of 50 years.
iv. Index patient's tumour is microsatellite-stable.

The M-FCCTX criterion eliminates the requirement for a colorectal cancer diagnosis before 50 years of age. Index patients not fulfilling high-risk criteria, but who had at least one FDR affected by colorectal cancer were designated as intermediate-risk patients. Index patients with no FDRs affected by colorectal cancer were designated as low-risk patients. A family history of colorectal cancer was defined as having at least one FDR affected by colorectal cancer at any age, in addition to the index patient.

Patients and controls were also asked to complete personal history (PHQ) and food frequency (FFQ) questionnaires. Participants were asked about demographic factors (age, sex, and highest education attained), anthropometric variables (height and weight), and medical history. Participants were asked about their use of alcohol, tobacco, non-steroidal anti-inflammatory drugs, dietary supplements (i.e. multivitamin, calcium and folate) and about their weekly dietary consumption of meat, fruit and vegetables. Their level of physical activity and whether they participated in colorectal cancer screening was also investigated. Women were asked about their menstrual status, as well as their use of hormone contraceptives and hormone replacement therapy (HRT).

This thesis utilized two different study designs in order to meet its objectives. A cohort study design was used to investigate the risk of cancer in families of patients defined as high-risk and intermediate-risk, as well as in patient subgroups defined by specific clinical and molecular characteristics. This thesis also utilized a case-control study design to investigate the association between certain dietary / lifestyle factors and colorectal cancer. Ethics approval for this research project was obtained from the Human Investigation Committee of Memorial University.

3.2 Statistical Analysis

Descriptive analyses were conducted and comparison of continuous variables was analyzed by either independent samples t-test or one-way ANOVA. Categorical variables were analyzed with either Fisher's exact test or Pearson's chi-square test. Cox proportional hazard models estimated hazard ratios (HR)

for developing cancer in first-degree relatives. Index patients were excluded from the estimates of cancer risk. Age at diagnosis or age at last follow-up was used as the time variable. Log minus log test of proportionality was used to test the assumption of proportional hazards. The sex of first-degree relatives (FDRs) was entered as a stratification variable. For families satisfying the FCCTX and M-FCCTX criteria, the unadjusted estimate of cancer risk was based on all first-degree relatives and excluded the index patient. The adjusted estimate excluded the affected relatives who were necessary to satisfy the family history criteria. To ensure consistency, selection of affected family members for exclusion was determined by identifying the earliest affected relatives that satisfied the FCCTX criteria (triad of affected relatives). The index patient was always identified as one of the three affected.

The cumulative lifetime risk (<75 years of age) for developing colorectal cancer in first-degree relatives was estimated by Kaplan-Meier survival analysis. Standardized incidence ratios (SIR) for CRC in FDRs was calculated using PAMCOMP software [286]. The SIR compares the incidence of CRC in the study cohort to a population-based cohort that was obtained from the Surveillance, Epidemiology, and End-Results (SEER) 9 cohort [287]. The SIR is obtained by dividing the number of observed cases of CRC by the "expected" number of cases. Index patients were excluded from the estimate of cancer risk in family members.

A binary logistic regression model was utilized to identify clinicopathological features that were associated with familial colorectal cancer patients. The association between clinicopathological features and patients who were stratified

by the molecular features of their tumour was estimated using odds ratios (OR) that were calculated from multinomial logistic regression models.

Comparison between controls and colorectal cancer patients stratified by the *V600E BRAF* mutation was estimated using odds ratios that were calculated from univariate multinomial logistic regression models. The association between diabetes, smoking, NSAIDs, BMI and colorectal cancer was estimated using adjusted odds ratios that were calculated from multinomial logistic regression models. Potential confounders were evaluated by testing their statistical significance in a univariate regression analysis. Variables that had a significance of $P<0.20$ were identified as potential confounders and were included in the multivariate models, regardless of their statistical significance in the final regression model. Missing values for continuous and categorical data were replaced by the sex-specific mean and mode, respectively, of the non-missing data. Statistical test for trend was calculated by entering the categorical exposure variable as a continuous variable into the logistic regression model.

All P values were two-sided and $P \leq 0.05$ was considered significant. All analyses were performed with the Predictive Analytics Statistics Software (PASW) package, version 18.0 (Chicago, IL).

Chapter 4 – Results

4.1 The Study Population

Ascertainment of the study population is depicted in **Figure 1**. Written consent had been obtained from 64% (n = 750) of eligible colorectal caner patients. Consenting patients were excluded: if they had a personal or family history consistent with familial adenomatous polyposis (n = 7); if their family history was non-informative (i.e. missing critical information to evaluate family history) (n = 116); or if the microsatellite-instability (n = 40) or *V600E BRAF* mutation status (n = 34) of their tumour was unknown. The final study population (n = 553) represented 47% of all eligible and consenting colorectal cancer patients.

Eligible study controls were contacted (n = 2,168) and 74% consented (n = 1,603) to participate in the study. Controls were excluded if they did not return a completed personal history questionnaire (n = 890). The final control study population (n = 713) represents 33% of eligible and consenting controls.

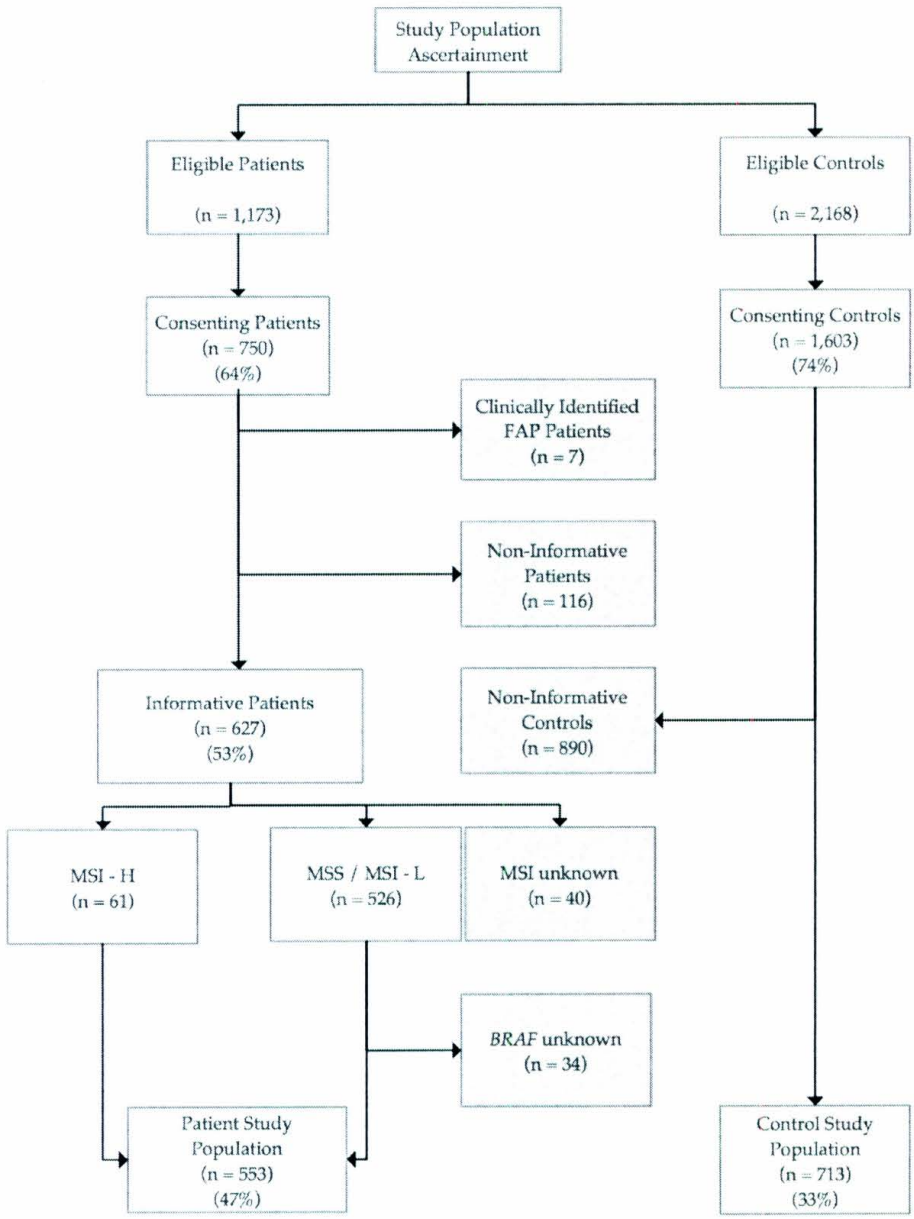

Figure 1 Ascertainment of the study population.

4.2 Epidemiology

Index patients were stratified according to the microsatellite-instability and the *V600E BRAF* mutation (*V600E*) status of their tumour, as well as according to whether they were affected by Lynch syndrome (**Figure 2**). The majority of patient tumours were MSS (89%) and the remainder MSI-H (11%). The prevalence of the *V600E* mutation in the study population was 11.8% (n = 65). In MSS tumours, the incidence of the *V600E* mutation was approximately 8%, whereas in MSI tumours it was 44%. Amongst MSI-H tumours, 17 were associated with patients who had a pathogenic MMR gene variant, 27 harbored the *V600E BRAF* mutation and the remaining 17 were *BRAF* Wt. The molecular pathology classification was unclear for these remaining 17 patient tumours (shaded in **Figure 2**), and these patients were excluded from further analysis. Tumours from index patients identified as "possible Lynch syndrome" (n = 9) are deficient in at least one MMR protein on IHC and the *MLH1* promoter was unmethylated (data not shown). Patients identified as "possible serrated pathway" contained a colorectal tumour that was hypermethylated at the *MLH1* promoter.

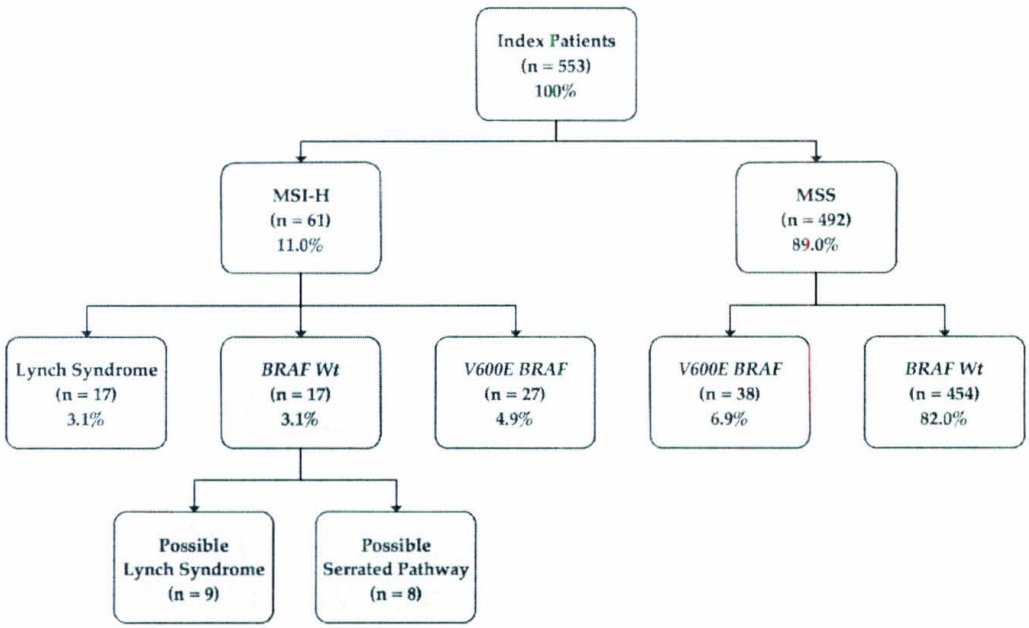

Figure 2 Classification of the study population according to molecular pathology.

A descriptive analysis of the clinicopathological features of index patients and their family history is presented in **Table 4.1.1**. The mean age of diagnosis for index patients is 61 years, and patients were more likely to be male (60.3%), and their tumours were more likely to be located in the distal colon or rectum (57.0%) rather than the proximal colon. Family history was evaluated by several criteria. The proportion of index patients who had history of colorectal cancer (i.e. $\geq$ 1 FDRs affected by colorectal cancer) was approximately 32%. A much

smaller proportion of the study population had a family history that satisfied the Amsterdam 1 criteria (4.9%). Amongst those who had a family history satisfying the Amsterdam I criteria (n = 27), 15 had a microsatellite-stable tumour and were unaffected by Lynch syndrome, thus satisfying the criteria for FCCTX. These FCCTX patients accounted for 56% of patients meeting the Amsterdam I criteria, and for approximately 3% of the entire study population. In addition to patients who satisfied the Amsterdam I criteria, we identified an additional 16 high-risk patients, identified as M-FCCTX, who had a family history consistent with autosomal dominant disease, but who did not satisfy the Amsterdam I criteria because of the age criterion (i.e. at least 1 FDR affected by CRC less than 50 years of age).

Table 4.1.1 Description and comparison of index patients according to the molecular status of their tumour.

Features	Molecular[a] Status of Tumuor		
	Known (n = 553)	Unknown (n = 74)	P^b
	No. (%)	*No. (%)*	
Clinicopathological variabels			
Mean age Dx (SD), yrs	61.0 (±9.1)	61.9 (±9.2)	0.41
Sex			0.25
Male	333 (60.3)	50 (67.6)	
Female	250 (39.7)	24 (32.4)	
Multiple Tumours			0.29
No	498 (90.1)	70 (94.6)	
Yes	55 (9.9)	4 (5.4)	
Tumor Location			<0.001
Distal Colon or Rectum	315 (57.0)	29 (39.2)	
Proximal Colon	223 (40.3)	12 (16.2)	
Unknown	15 (2.7)	33 (44.6)	
Clinical Stage of disease			<0.001
Local disease (Stage 1 & 2)	225 (40.7)	24 (32.4)	
Advanced Disease (Stage 3 & 4)	268 (50.3)	12 (16.2)	
Unknown	60 (10.8)	36 (48.6)	
Pathological Variables			
Tumour Grade			<0.001
Well or Moderately Differentiated	494 (89.3)	39 (52.7)	
Poorly Differentiated	43 (7.8)	1 (1.4)	
Unknown	16 (2.9)	34 (45.9)	
Crohn's-like Lymphoid Reaction			<0.001
Absent	223 (40.3)	21 (28.4)	
Present	311 (56.2)	20 (27.0)	
Unknown	19 (3.4)	33 (44.6)	
Tumor Infiltrating Lymphocytes			<0.001
Absent	392 (70.9)	32 (43.2)	
Present	146 (26.4)	9 (12.2)	
Unknown	15 (2.7)	33 (44.6)	
Mucinous Component			<0.001
Absent	395 (71.4)	35 (47.3)	
Present	143 (25.9)	6 (8.1)	
Unknown	15 (2.7)	33 (44.6)	
Family History Variables			
Amsterdam I Criteria (AC-1)			0.04
No	526 (95.1)	74 (100)	
Yes	27 (4.9)	0 (0)	
FCCTX Criteria			0.38
No	538 (97.3)	74 (100)	
Yes	15 (2.7)	0 (0)	
M-FCCTX Criteria			0.24
No	537 (97.1)	74 (100)	
Yes	16 (2.9)	0 (0)	
≥1 FDR affected by CRC			0.35
No	374 (67.6)	54 (73.0)	
Yes	179 (32.4)	20 (27.0)	
Molecular Variables			
MMR Mutation			na
No	536 (96.9)	74 (100)	
Yes	17 (3.1)	0 (0)	
Microsatellite-Instability			na
MSS	492 (89.0)	34 (45.9)	
MSI	61 (11.0)	0 (0)	
Unknown	0 (0)	40 (54.1)	
BRAF			na
Wild Type (Wt)	488 (88.2)	6 (8.1)	
V600E	65 (11.8)	0 (0)	
Unknown	0 (0)	68 (91.9)	

MSS = microsatellite-stable; MSI = microsatellite-unstable; *na* = not applicable

[a] *V600E BRAF* mutation and microsatellite-instability status of tumour.

[b] *P* value determined by either Chi-square or Fisher's exact test.

Patients were excluded from this study if the molecular pathology (i.e. MSI or *V600E* status) of their tumour was unknown (n = 74). A description and comparative analysis of these patients is also presented in **Table 4.1.1.** Patients who were excluded were not significantly different from study participants with respect to family history ($P = 0.35$), which is the primary outcome under investigation. However, excluded patients were found to be significantly different with respect to other variables.

4.2 Inherited Colorectal Cancer

4.2.1 High-Risk Patients

Approximately 9% (n = 48) of all index patients were identified as high-risk patients and were either affected by Lynch syndrome (n = 17) or satisfied the FCCTX (n= 15) or M-FCCTX criteria (n = 16). A descriptive and comparative analysis of these patients is presented in **Table 4.2.1**. Lynch syndrome patients are characterized by having an early age diagnosis of colorectal cancer (mean = 51 yrs) and a high incidence (41%) of multiple tumours (i.e. a synchronous or metachronous colorectal tumour).

The phenotype associated with FCCTX patients was significantly different when compared to Lynch syndrome patients. FCCTX patients were significantly older at diagnosis ($P = 0.02$), less likely to have multiple tumours ($P = 0.04$) and more likely to have a tumour located in the distal rather than proximal colon (64% vs. 47%), although this result was not statistically significant ($P = 0.46$). The clinicopathological phenotype associated with M-FCCTX, however, was similar to FCCTX. These two patient subgroups were not statistically different with

respect to the occurrence of multiple tumours ($P = 0.48$), tumour location ($P = 0.27$) or stage of disease ($P = 0.57$). Patients identified as M-FCCTX were, however, slightly older at diagnosis than FCCTX patients (64 yrs vs. 59 yrs), but the result did not reach statistical significance ($P = 0.12$).

Table 4.2.1 Comparison of clinicopathological features between high-risk colorectal cancer patients.

	Lynch syndrome n = 17	**FCCTX** n = 15	P^a	**M-FCCTX** n = 16	P^b
	No. (%)	*No. (%)*		*No. (%)*	
Mean age Dx (SD), yrs	51.1 (±10.3)	59.3 (±8.7)	0.02	63.9 (±7.0)	0.12
Sex					
Male	12 (70.6)	11 (73.3)	1.00	11 (68.8)	1.00
Female	5 (29.4)	4 (26.7)		5 (31.3)	
Multiple Tumours					
No	10 (58.8)	14 (93.3)	0.04	16 (100)	0.48
Yes	7 (41.2)	1 (6.7)		0 (0)	
Tumour Location					
Distal Colon & Rectum	7 (46.7)	9 (64.3)	0.46	6 (37.5)	0.27
Proximal Colon	8 (53.3)	5 (35.7)		10 (62.5)	
Clinical Stage of Disease					
Local Disease (Stages 1 & 2)	6 (50.0)	7 (53.8)	0.85	6 (42.9)	0.57
Advanced Disease (Stages 3 & 4)	6 (50.0)	6 (46.2)		8 (57.1)	
Microsatellite-Instability					
MSS	0 (0)	15 (100)	*na*	16 (100)	*na*
MSI	17 (100)	0 (0)		0 (0)	
***BRAF* Mutation**					
Wild Type	12 (100)	13 (86.7)	0.49	16 (100)	0.23
V600E	0 (0)	2 (13.3)		0 (0)	

MSS = microsatellite-stable; MSI = microsatellite-unstable; *na* = not applicable

[a] Comparison between Lynch syndrome and FCCTX patients. P value determined by either chi-sqaure or fisher's exact test.

[b] Comparison between FCCTX and M-FCCTX patients. P value determined by either chi-sqaure or fisher's exact test.

We investigated and compared the risk of developing colorectal cancer in the FDRs of high-risk patients with time-to-event analyses (**Table 4.2.2**). The lifetime risk for developing colorectal cancer was greatest in the FDRs of Lynch syndrome patients. The lifetime risk for developing colorectal cancer in FDRs of Lynch syndrome patients was estimated to be approximately 50% (LR% = 52%; 95% CI, 38% - 66%). When compared to Lynch syndrome families, the risk of colorectal cancer in FCCTX families was less (LR% = 35%; 95% CI, 23% – 47%), but the difference was not statistically significant (logrank $P = 0.07$). However, the risk in M-FCCTX families was significantly less when compared to Lynch syndrome families (LR% = 39%; 95% CI, 25% – 53%; logrank $P = 0.003$).

Table 4.2.2 The risk of colorectal cancer in first-degree relatives of high-risk colorectal cancer patients.

Classification	Index Patients	FDRs at-risk	CRC Events	Risk of Colorectal Cancer			
	No.	*No.*	*No.*	*LR[b] (95% CI)*	*SIR (95% CI)*	*HR[c] (95% CI)*	*P*
Lynch syndrome	17	154	35	52 (38 - 66)	27.3 (18.9 - 38.2)	1.00 (Ref.)	
FCCTX	15	163	33	35 (23 - 47)	15.2 (10.5 - 21.3)	-	
FCCTX[a]	15	139	9	13 (3 - 23)	4.9 (2.3 - 9.4)	0.20 (0.10 - 0.41)	**<0.001**
M-FCCTX	16	182	29	39 (25 - 53)	12.5 (8.3 - 17.9)	-	
M-FCCTX[a]	16	160	7	14 (4 - 24)	4.4 (1.8 - 9.0)	0.16 (0.07 - 0.36)	**<0.001**

FDRs = first-degree relatives; HR = hazard ratio; LR = Lifetime risk (<75 years of age); SIR = standardized incidence ratio

[a] Adjusted estimate: affected family members who are necessary to fulfill the FCCTX and M-FCCTX criteria were excluded.

[b] Lifetime risk estimated by kaplan-Meier survival analysis.

[c] Hazard ratio estimated by Cox Regression model.

Figure 3 demonstrates that the greater risk of developing colorectal caner in Lynch syndrome families is largely attributable to the occurrence of early-age cancers (i.e. diagnosis < 50 years of age) in these families. This is also true for families identified as FCCTX. However, **Figure 3** also demonstrates that the cancer risk profiles for these 3 patients subgroups is very similar after the age of 50 years.

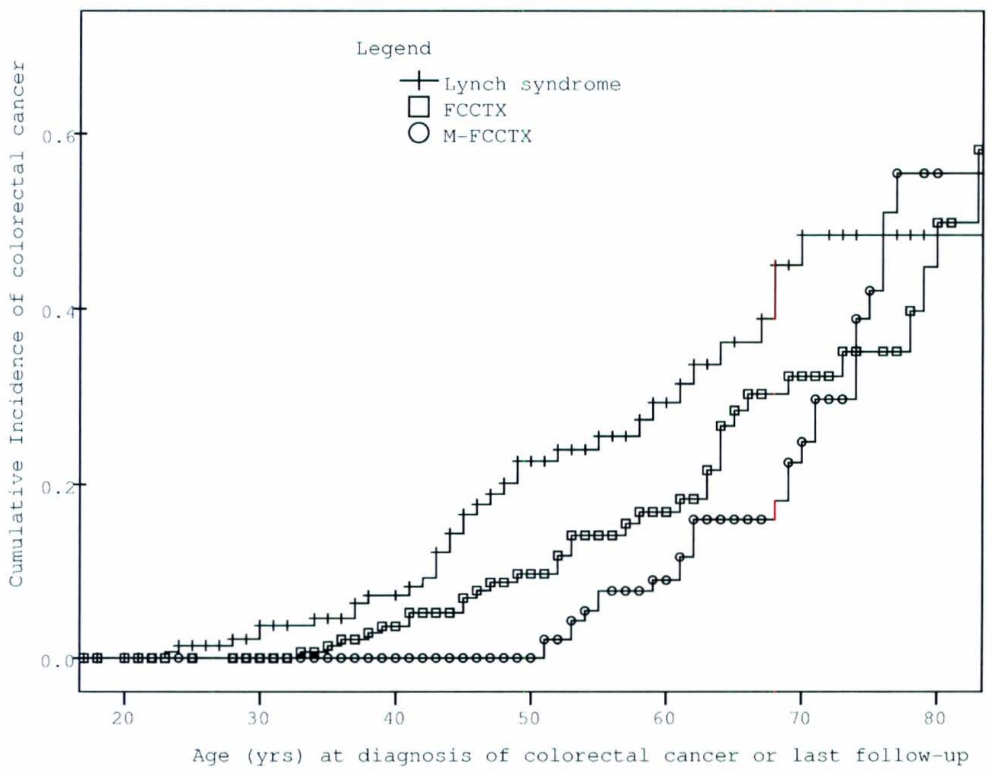

Figure 3 The cumulative incidence of colorectal cancer in first-degree relatives of high-risk patients.

To adjust for the selection bias introduced by comparing the risk of colorectal cancer in families identified by a genetic mutation (Lynch syndrome) to families identified by family history criteria (FCCTX and M-FCCTX), we excluded from the analysis affected family members in FCCTX and M-FCCTX families who were necessary to satisfy the family history criteria. The adjusted relative risk estimate for developing colorectal cancer in FDRs of patients identified as FCCTX (HR = 0.20; 95% CI, 0.10 – 0.41; $P < 0.001$) and M-FCCTX (HR = 0.16; 95% CI, 0.07 – 0.36; $P < 0.001$) was significantly lower when compared to Lynch syndrome families (**Table 4.2.2**).

4.2.2 Intermediate-Risk Patients

Index patients who did not fulfill high-risk criteria and did not contain a MMR mutation (n = 488), 26% (n = 127), but who had at least one FDR affected by colorectal cancer, were designated as intermediate-risk patients (**Table 4.3.1**). Index patients without a FDR affected by colorectal cancer were designated as low-risk patients (n = 361). We investigated the association between family history and clinicopathological features and molecular markers of carcinogenesis in non-high-risk patients (**Table 4.3.1**). In univariate analysis, moderate-risk patients were more likely to have multiple tumours ($P = 0.01$), a tumour located in the proximal colon ($P = 0.08$), and more likely to have a MSI-H tumour ($P = 0.001$) and a *V600E* tumour ($P <0.001$) than low-risk patients. Moderate-risk patients were also slightly older at diagnosis, but the result was not statistically significant (P trend = 0.15). However, in multivariate analysis only two features remained

significantly associated with moderate-risk patients – the occurrence of multiple tumours (odds ratio = 1.98; 95% CI, 1.02 – 3.84; $P = 0.04$) and the presence of the *V600E* mutation (odds ratio = 2.71; 95% CI, 1.56 – 4.71; $P < 0.001$).

Table 4.3.1 Clinicopathological features associated with non high-risk colorectal cancer patients who have a family history of colorectal cancer.

Feature	Low-Risk[a]	Intermediate-Risk[b]	Univariate	Multivariate[c]
	n = 361 *No. (%)*	n = 127 *No. (%)*	*OR[d] (95% CI)*	*OR (95% CI)*
Age				
≤55	95 (26.3)	27 (21.3)	1.00 (Ref.)	
56 - 65	132 (36.6)	43 (33.9)	1.08 (0.62 - 1.89)	ns
>65	134 (37.1)	57 (44.9)	1.44 (0.84 - 2.44)	
P trend			0.15	
Sex				
Male	219 (60.7)	70 (55.1)	1.00 (Ref.)	
Female	142 (39.3)	57 (44.9)	1.25 (0.83 - 1.89)	ns
P			0.30	
Multiple Tumour				
No	336 (93.1)	109 (85.8)	1.00 (Ref.)	1.00 (Ref.)
Yes	25 (6.9)	18 (14.2)	2.24 (1.17 - 4.26)	1.98 (1.02 - 3.84)
P			**0.01**	**0.04**
Tumour Location				
Distal Colon or Rectum	223 (63.0)	67 (54.0)	1.00 (Ref.)	
Proximal Colon	131 (37.0)	57 (46.0)	1.45 (0.96 - 2.19)	ns
P			0.08	
MSI				
MSS	349 (96.7)	112 (88.2)	1.00 (Ref.)	
MSI	12 (3.3)	15 (11.8)	3.92 (1.78 - 8.63)	ns
P			**0.001**	
***BRAF* Mutation**				
Wild Type	327 (90.6)	98 (77.2)	1.00 (Ref.)	1.00 (Ref.)
V600E	34 (9.4)	29 (22.8)	2.87 (1.67 - 4.96)	2.71 (1.56 - 4.71)
P			**<0.001**	**<0.001**

OR = odds ratio; ns = non-significant

[a] Low-Risk patients have no family history of colorectal cancer in first-degree relatives.

[b] Intermediate-Risk patients have ≥ 1 first-degree relatives affected by colorectal cancer.

[c] Forward conditional binary logistic regression model

[d] OR estimated by binary logistic regression model.

We stratified non-high-risk patients according to the *V600E* mutation status of their tumour and according to whether or not the patient had multiple tumours. In the event that a patient had both a *V600E* tumour and multiple tumours (n = 3), it was decided that the *V600E* mutation would take precedence. Accordingly, 63 patients who had a *V600E* tumour were designated as "*V600E*", 32 patients who had multiple tumors (*BRAF* Wt) were designated as "multiple tumours", and patients without either of these features were designated as "remaining" (n = 393). We compared the risk of developing colorectal cancer in FDRs between these 3 patient subgroups (**Table 4.3.2**). The hazard for developing colorectal cancer was significantly greater in FDRs of patients designated as either "multiple tumours" (HR = 1.88; 95% CI, 1.11 – 3.20; $P = 0.02$) or "*V600E*" (HR = 2.66; 95% CI, 1.83 – 3.86; $P < 0.001$), when compared to "remaining" families.

Table 4.3.2 The risk of colorectal cancer in first-degree relatives of non high-risk patients stratified by the *V600E BRAF* mutation and the occurrence of a multiple tumours.

Tumour Pathology	Index Patients	FDRs at-risk	CRC Events	Risk of Colorectal Cancer		
	No.	*No.*	*No.*	*LR%*[a] *(95% CI)*	*HR*[b] *(95% CI)*	*P*
V600E BRAF (MSS or MSI-H tumour)	63	632	39	15 (10 - 20)	2.66 (1.83 - 3.86)	**<0.001**
Multiple Tumours (MSS & *BRAF* Wt tumour)	32	300	16	11 (5 - 17)	1.88 (1.11 - 3.20)	**0.02**
Remainaing (MSS & *BRAF* Wt tumour)	393	3728	94	6 (5 - 7)	1.00 (Ref.)	

FDR = first-degree relative HR = hazard ratio; LR = Lifetime Risk (<75 years of age)

[a] Lifetime risk estimated by kaplan-Meier survival analysis.

[b] Hazard ratio estimated by Cox Regression model.

The cumulative incidence of colorectal cancer in FDRs according to the designation of the index patient is presented in **Figure 4.** This figure demonstrates that the incidence of colorectal cancer in FDRs of "multiple tumors" and "*V600E*" is greater, particularly after the age of 50 years, than in the family members of patients identified as "remaining".

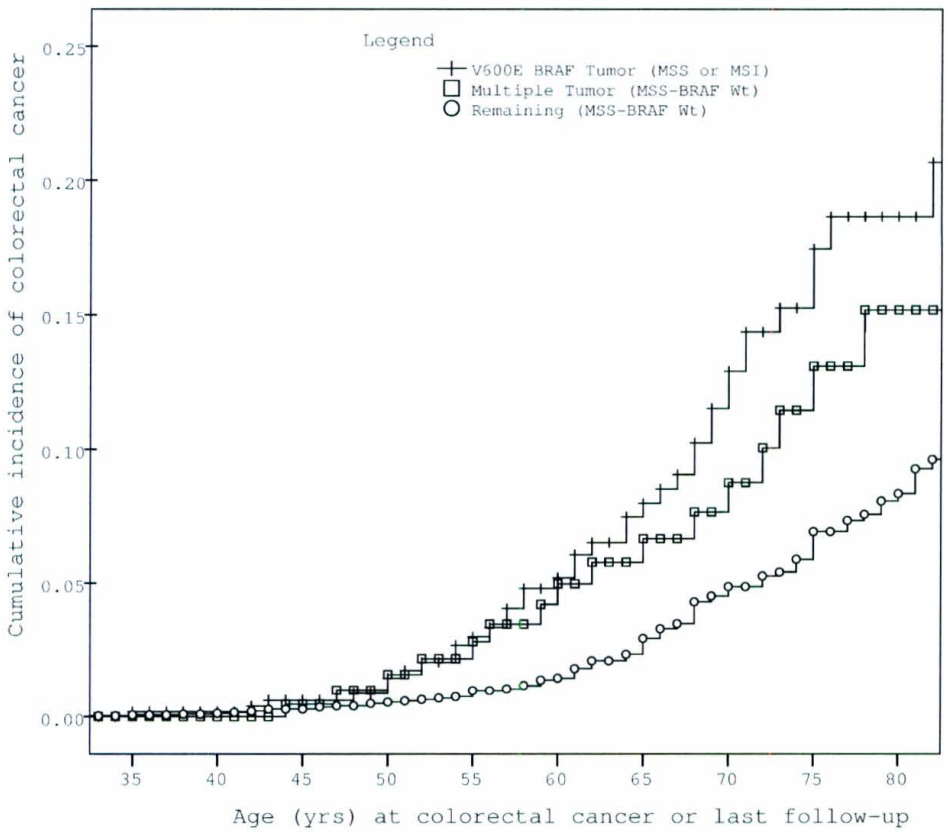

Figure 4 The cumulative incidence of colorectal cancer in first-degree relatives of non high-risk patients stratified by the *V600E BRAF* status of their tumour and the occurrence of multiple tumours. Abbreviations: MSS = microsatellite-stable; MSI = microsatellite-instability high

A comparison of the clinicopathological features of patients identified as "multiple tumours", "*V600E*" and "remaining" patients was investigated with a multinomial logistic regression analysis (**Table 4.3.3)**. Compared to "remaining" patients, "*V600E*" patients exhibited a distinct phenotype and were significantly more likely to be female ($P = 0.004$) and to have a tumour located in the proximal colon ($P < 0.001$). Additionally, their colorectal tumours were more likely to be poorly differentiated ($P = 0.02$), to contain tumour-infiltrating lymphocytes ($P = 0.01$) and to have a mucinous component ($P < 0.001$). In contrast, patients designated "multiple tumours" did not exhibit any clinicopathological differences when compared to "remaining" patients.

Table 4.3.3 Comparison of clinicopathological features of non high-risk patients, stratified by the *V600E BRAF* mutation and the occurrence of multiple tumours.

Clinicopathological Feature	MSS-*BRAF* Wt	*V600E BRAF* (MSS & MSI-H)			Multiple Tumours (MSS-*BRAF* Wt)		
	n = 393	n = 63			n = 32		
			Univariate	Multivariate		Univariate	Multivariate
	No. (%)	*No. (%)*	*OR[a] (95% CI)*	*AOR (95% CI)*	*No. (%)*	*OR (95% CI)*	*AOR (95% CI)*
Age							
≤55	106 (27.0)	9 (14.3)	1.00 (Ref.)	1.00 (Ref.)	7 (21.9)	1.00 (Ref.)	1.00 (Ref.)
56 - 65	138 (35.1)	28 (44.4)	2.39 (1.08 - 5.28)	1.62 (0.63 - 4.13)	9 (28.1)	0.99 (0.36 - 2.74)	0.88 (0.28 - 2.75)
>65	149 (37.9)	26 (41.3)	2.06 (0.93 - 4.56)	1.94 (0.77 - 4.92)	16 (50.0)	1.63 (0.65 - 4.09)	1.95 (0.71 - 5.37)
P-trend			0.14	0.18		0.24	0.17
Sex							
Male	250 (63.6)	22 (34.9)	1.00 (Ref.)	1.00 (Ref.)	17 (53.1)	1.00 (Ref.)	1.00 (Ref.)
Female	143 (36.4)	41 (65.1)	3.26 (1.87 - 5.69)	2.72 (1.38 - 5.33)	15 (46.9)	1.54 (0.75 - 3.18)	1.55 (0.70 - 3.44)
P			**<0.001**	**0.004**		0.24	0.28
Tumour Location							
Distal Colon or Rectum	261 (68.1)	8 (12.7)	1.00 (Ref.)	1.00 (Ref.)	21 (65.6)	1.00 (Ref.)	1.00 (Ref.)
Proximal Colon	122 (31.9)	55 (87.3)	14.71 (6.80 - 31.84)	8.94 (3.97 - 20.18)	11 (34.4)	1.12 (0.52 - 2.40)	1.10 (0.48 - 2.50)
P			**<0.001**	**<0.001**		0.77	0.82
Clinical Stage of Disease							
Local Disease (Stages 1 & 2)	161 (45.4)	23 (39.7)	1.00 (Ref.)	1.00 (Ref.)	12 (42.9)	1.00 (Ref.)	1.00 (Ref.)
Advanced Disease (Stages 3 & 4)	194 (54.6)	35 (60.3)	1.26 (0.72 - 2.22)	1.19 (0.60 - 2.38)	16 (57.1)	1.11 (0.51 - 2.41)	1.26 (0.56 - 2.80)
P			0.42	0.62		0.80	0.58
Tumour Grade							
Well or Moderately Differentiated	359 (93.7)	49 (77.8)	1.00 (Ref.)	1.00 (Ref.)	31 (96.9)	1.00 (Ref.)	1.00 (Ref.)
Poorly Differentiated	24 (6.3)	14 (22.2)	4.27 (2.07 - 8.81)	2.91 (1.07 - 7.93)	1 (3.1)	0.49 (0.06 - 3.69)	0.52 (0.07 - 4.09)
P			**<0.001**	**0.04**		0.48	0.53
Crohn's-Like Reaction							
Absent	165 (43.5)	23 (36.5)	1.00 (Ref.)	1.00 (Ref.)	17 (53.1)	1.00 (Ref.)	1.00 (Ref.)
Present	214 (56.5)	40 (63.5)	1.34 (0.77 - 2.33)	1.46 (0.74 - 2.89)	15 (46.9)	0.68 (0.33 - 1.40)	0.77 (0.35 - 1.69)
P			0.30	0.28		0.30	0.52
Tumor-Infiltrating Lymphocytes							
Absent	298 (77.8)	35 (55.6)	1.00 (Ref.)	1.00 (Ref.)	23 (71.9)	1.00 (Ref.)	1.00 (Ref.)
Present	85 (22.2)	28 (44.4)	2.81 (1.61 - 4.87)	2.36 (1.19 - 4.68)	9 (28.1)	1.37 (0.61 - 3.08)	1.54 (0.66 - 3.63)
P			**<0.001**	**0.01**		0.44	0.32
Mucinous Component							
Absent	303 (79.1)	29 (46.0)	1.00 (Ref.)	1.00 (Ref.)	25 (78.1)	1.00 (Ref.)	1.00 (Ref.)
Present	80 (20.9)	34 (54.0)	4.44 (2.55 - 7.72)	3.60 (1.84 - 7.05)	7 (21.9)	1.06 (0.44 - 2.54)	1.04 (0.40 - 2.71)
P			**<0.001**	**<0.001**		0.90	0.94

OR = odds ratio; AOR = adjusted OR

[a] OR estimated by multinomial logistic regression model.

4.3 V600E Colorectal Cancer

To investigate the impact of MSI status in *V600E* colorectal cancer, index patients were stratified according to the microsatellite-instability and *V600E* status of their tumour, as follows:

i) MSS-*BRAF* Wt tumour (n = 454)

ii) MSS-*V600E* tumour (n = 38)

iii) MSI-*V600E* tumour (n = 27)

The clinicopathological differences between patients stratified according to the MSI and *V600E* status of their tumours was investigated with a multinomial regression analysis (**Table 4.4.1**). *V600E* colorectal cancer, when compared to MSS-*BRAF* Wt colorectal cancer, exhibited a distinct clinicopathological profile irrespective of MSI status. For example, *V600E* colorectal cancer (MSS and MSI tumours) was significantly associated with proximal tumour location ($P < 0.001$; for both groups) and with a mucinous component ($P = 0.02$ and $P = 0.001$, respectively) when compared to MSS-*BRAF* Wt colorectal cancer. While both MSS- and MSI-*V600E* colorectal cancer patients tended to be female, the result was statistically significant only for patients who had a MSS-*V600E* tumour ($P = 0.01$). However, some clinicopathological features were observed to be dependent on MSI status. For example, MSI-*V600E* colorectal cancer patients were significantly older at diagnosis ($P = 0.04$), more likely to have multiple tumors ($P = 0.009$) and their tumors were more likely to contain tumour-infiltrating lymphocytes ($P <0.001$), when compared to MSS-*BRAF* Wt colorectal

cancer; these features was not observed with MSS-*V600E* colorectal cancer. However, MSS-*V600E* colorectal cancer was significantly associated with poorly differentiated tumours ($P = 0.02$) when compared to MSS-*BRAF* Wt colorectal cancer.

Table 4.4.1 Comparison of clinicopathological features of patients stratified by the microsatellite-instability and *V600E BRAF* mutation status of their tumours.

Feature	MSS-*BRAF* Wt Tumour	MSS-*V600E* Tumour			MSI-*V600E* Tumour		
	n = 454	n = 38			n = 27		
	No. (%)	*No. (%)*	Univariate *OR[a] (95% CI)*	Multivariate *AOR (95% CI)*	*No. (%)*	Univariate *OR (95% CI)*	Multivariate *AOR (95% CI)*
Age							
≤65	278 (61.2)	28 (73.7)	1.00 (Ref.)	1.00 (Ref.)	10 (37.0)	1.00 (Ref.)	1.00 (Ref.)
>65	176 (38.8)	10 (26.3)	0.56 (0.27 - 1.19)	0.78 (0.34 - 1.79)	17 (63.0)	2.69 (1.20 - 6.00)	2.93 (1.07 - 8.02)
P			0.13	0.56		**0.02**	**0.04**
Sex							
Male	288 (63.4)	14 (36.8)	1.00 (Ref.)	1.00 (Ref.)	9 (33.3)	1.00 (Ref.)	1.00 (Ref.)
Female	166 (36.6)	24 (63.2)	2.97 (1.50 - 5.91)	2.67 (1.22 - 5.88)	18 (66.7)	3.47 (1.52 - 7.90)	2.70 (0.97 - 7.47)
P			**0.002**	**0.01**		**0.003**	0.06
Multiple Tumour							
No	421 (92.7)	34 (89.5)	1.00 (Ref.)	1.00 (Ref.)	20 (74.1)	1.00 (Ref.)	1.00 (Ref.)
Yes	33 (7.3)	4 (10.5)	1.50 (0.50 - 4.49)	1.46 (0.43 - 5.05)	7 (25.9)	4.47 (1.76 - 11.33)	5.56 (1.55 - 19.98)
P			0.47	0.55		**0.002**	**0.009**
Tumour Location							
Distal Colon or Rectum	297 (66.9)	7 (18.9)	1.00 (Ref.)	1.00 (Ref.)	1 (3.7)	1.00 (Ref.)	1.00 (Ref.)
Proximal Colon	147 (33.1)	30 (81.1)	8.66 (3.72 - 20.18)	5.74 (2.36 - 13.95)	26 (96.3)	52.53 (7.06 - 390)	45.0 (5.43 - 372)
P			**<0.001**	**<0.001**		**<0.001**	**<0.001**
Clinical Stage of Disease							
Local Disease (Stages 1 & 2)	185 (45.20	9 (26.5)	1.00 (Ref.)	1.00 (Ref.)	15 (60.0)	1.00 (Ref.)	1.00 (Ref.)
Advanced Disease (Stages 3 & 4)	224 (54.8)	25 (73.5)	2.29 (1.05 - 5.04)	1.91 (0.82 - 4.45)	10 (40..0)	0.55 (0.24 - 1.26)	0.37 (0.13 - 1.09)
P			**0.04**	0.14		0.16	0.07
Tumor Grade							
Well or Moderately Differentiated	417 (93.9)	27 (73.0)	1.00 (Ref.)	1.00 (Ref.)	23 (85.2)	1.00 (Ref.)	1.00 (Ref.)
Poorly Differentiated	27 (6.1)	10 (27.0)	5.72 (2.51 - 13.03)	3.34 (1.18 - 9.44)	4 (14.8)	2.69 (0.87 - 8.32)	1.99 (0.43 - 9.28)
P			**<0.001**	**0.02**		0.09	0.38
Crohn's-Like Reaction							
Absent	191 (43.4)	14 (40.5)	1.00 (Ref.)	1.00 (Ref.)	8 (29.6)	1.00 (Ref.)	1.00 (Ref.)
Present	249 (56.6)	22 (59.5)	1.13 (0.57 - 2.23)	1.22 (0.56 - 2.68)	19 (70.4)	1.82 (0.78 - 4.25)	1.42 (0.50 - 4.03)
P			0.74	0.62		0.17	0.51
Tumor-Infiltrating Lymphocytes							
Absent	344 (77.5)	26 (70.3)	1.00 (Ref.)	1.00 (Ref.)	10 (37.0)	1.00 (Ref.)	1.00 (Ref.)
Present	100 (22.5)	11 (29.7)	1.46 (0.70 - 3.05)	1.15 (0.49 - 2.70)	17 (63.0)	5.85 (2.60 - 13.18)	7.58 (2.54 - 22.59)
P			0.32	0.75		**<0.001**	**<0.001**
Mucinous Component							
Absent	348 (78.4)	20 (54.1)	1.00 (Ref.)	1.00 (Ref.)	10 (37.0)	1.00 (Ref.)	1.00 (Ref.)
Present	96 (21.6)	17 (45.9)	3.08 (1.55 - 6.11)	2.52 (1.16 - 5.48)	17 (63.0)	6.16 (2.73 - 13.90)	6.62 (2.26 - 19.43)
P			**0.001**	**0.02**		**<0.001**	**0.001**

OR = odds ratio; AOR = adjusted OR

[a] Odds ratio estimated by multinomial regression model.

Additionally, we have investigated the effect of tumour microsatellite-instability status on the association between family history and *V600E* colorectal cancer (**Table 4.4.2**). The lifetime risk of colorectal cancer in FDRs of index patients who had a MSS-*BRAF* Wt was estimated to be 9% (95% CI, 7% – 11%). Compared to this, the relative risk of colorectal cancer was significantly elevated in the FDRs of patients who had either a MSS-*V600E* tumour (HR = 1.75; 95% CI, 1.12 – 2.72; $P = 0.01$) or MSI-*V600E* tumour (HR = 2.18; 95% CI, 1.42 – 3.34; $P < 0.001$).

Table 4.4.2 The risk of colorectal cancer in first-degree relatives of index patients stratified by the microsatellite-instability and *V600E BRAF* mutation status of their tumour.

Tumour Pathology	Index Patients	FDRs	CRC Events	Risk of Developing Colorectal Cancer		
	No.	*No.*	*No.*	*LR%*[a] *(95% CI)*	*HR*[b] *(95% CI)*	*P Value*
MSI-*V600E BRAF*	27	290	24	18 (10 - 26)	2.18 (1.42 - 3.34)	**<0.001**
MSS-*V600E BRAF*	38	370	22	15 (8 - 22)	1.75 (1.12 - 2.72)	**0.01**
MSS-*BRAF* Wt	454	4345	165	9 (7 - 11)	1.00 (Ref.)	

FDR = first-degree relatives; LR = lifetime risk; HR = hazard ratio

[a] Lifetime risk estimated by kaplan-Meier survival analysis.

[b] Hazard ratio estimated by Cox Regression model.

The risk of extracolonic malignancies in FDRs of index patients stratified according to MSI and *V600E* mutation status was also investigated (**Table 4.4.3**). The incidence of non-melanoma skin cancer was significantly elevated in the FDRs of MSS-*V600E* colorectal cancer (HR = 2.61; 95% CI, 1.15 – 5.92) and MSI-*V600E* colorectal cancer patients (HR = 2.81; 95% CI, 1.07 – 7.41) when compared to FDRs of MSS-*BRAF* Wt colorectal cancer patients. The incidence of breast, lung and prostate cancer in family members did not differ between these patients groups.

Table 4.4.3 The risk of extracolonic tumours in first-degree relatives of index patients stratified by the microsatellite-instability and *V600E BRAF* mutation status of their tumour.

Tumour Pathology	FDRs at-risk	Risk of Breast Cancer		Risk of Lung Cancer		Risk of Skin[a] Cancer		Risk of Prostate Cancer	
	No.	*No.*	*HR[b] (95% CI)*	*No.*	*HR (95% CI)*	*No.*	*HR (95% CI)*	*No.*	*HR (95% CI)*
MSI-*V600E BRAF*	290	4	1.14 (0.41 - 3.20)	2	0.52 (0.13 - 2.16)	5	2.81 (1.07 - 7.41)	1	0.65 (0.09 - 4.84)
MSS-*V600E BRAF*	370	3	0.71 (0.22 - 2.26)	6	1.15 (0.49 - 2.66)	7	2.61 (1.15 - 5.92)	4	1.41 (0.50 - 3.98)
MSS-*BRAF* Wt	4345	65	1.00 (Ref.)	67	1.00 (Ref.)	37	1.00 (Ref.)	38	1.00 (Ref.)

FDR = first-degree relatives; HR = Hazard Ratio

[a] non-melanoma skin cancer.

[b] hazard ratio estimated by Cox Regression model.

To evaluate the mode of disease inheritance in families of patients with specific molecular pathology, we compared the incidence of colorectal cancer between parents and siblings (**Table 4.4.4**). The relative risk of colorectal cancer was significantly greater in siblings when compared to parents (HR = 3.28; 95% CI, 1.09 – 9.89) for patients who had a MSS-*V600E* tumour. However, this was not observed for patients who had a MSI-*V600E* tumour (HR = 1.23; 95% CI, 0.65 – 3.06).

Table 4.4.4 The risk of colorectal cancer in parents and siblings of index patients stratified by the microsatellite-instability and *V600E BRAF* mutation status of their tumour.

Tumour Pathology	**Type of FDR**	**FDRs at-risk**	**CRC Events**	**Risk of Colorectal Cancer**
		No.	*No.*	*HR[a] (95% CI)*
MSI-*V600E BRAF*	Parent	48	8	1.00 (Ref.)
	Sibling	135	14	1.23 (0.65 – 3.06)
MSS-*V600E BRAF*	Parent	71	5	1.00 (Ref.)
	Sibling	151	13	3.28 (1.09 – 9.89)
MSS-*BRAF* Wt	Parent	866	67	1.00 (Ref.)
	Sibling	2047	83	1.61 (1.14 – 2.28)

FDR = first-degree relative; HR = hazard ratio

[a] Hazard ratio estimated by Cox Regression model.

4.4 Epidemiological Factors and V600E Colorectal Cancer

A description and comparison of colorectal cancer patients and controls is presented in **Table 4.5.1**. *BRAF* Wt colorectal cancer patients accounted for 87% (n = 436) of the patient study population. Compared to controls, *BRAF* Wt colorectal cancer patients were significantly less likely to have a college education ($P < 0.001$), to participate in colorectal cancer screening ($P < 0.001$), and to take calcium supplements ($P = 0.005$), but had a significantly greater fiber intake ($P < 0.001$). *V600E* colorectal cancer patients accounted for the remainder of patients (13%; n = 63) and were significantly older at diagnosis ($P = 0.01$) and less educated ($P = 0.002$) than controls, but had a greater fiber intake ($P = 0.01$) and were more likely to be female ($P < 0.001$). Additionally, female *V600E* patients were significantly less likely to have ever used hormone contraceptives ($P < 0.001$) and hormone replacement therapy ($P = 0.003$) than controls, but were more likely to be post-menopausal (100% vs. 87%).

Table 4.5.1 Description and comparison of controls and patients who are stratified by the *V600E BRAF* mutation status of their tumour.

Factor	Controls	*BRAF* Wt Tumour		*V600E BRAF* Tumour	
	n = 717	n = 436		n = 63	
	No. (%)	*No. (%)*	*OR[a] (95% CI)*	*No. (%)*	*OR (95% CI)*
Age					
<57 yrs	239 (33.3)	147 (33.7)	1.00 (Ref.)	13 (20.6)	1.00 (Ref.)
57 - 66 yrs	257 (35.9)	142 (32.6)	0.90 (0.67 - 1.20)	25 (39.7)	1.79 (0.89 - 3.58)
>66 yrs	221 (30.8)	147 (33.7)	1.08 (0.81 - 1.45)	25 (39.7)	2.08 (1.04 - 4.17)
P trend			0.96		**0.04**
Sex					
Male	424 (59.1)	274 (62.8)	1.00	22 (34.9)	1.00
Female	293 (40.9)	162 (37.2)	0.86 (0.67 - 1.09)	41 (65.1)	2.70 (1.57 - 4.62)
P			0.21		**<0.001**
Education					
≤High School	349 (48.7)	275 (63.1)	1.00	44 (69.8)	1.00
>High School	368 (51.3)	161 (36.9)	0.56 (0.44 - 0.71)	19 (30.2)	0.41 (0.23 - 0.72)
P			**<0.001**		**0.002**
Any CRC Screening					
No	566 (78.9)	394 (90.4)	1.00	51 (81.0)	1.00
Yes	151 (21.1)	42 (9.6)	0.40 (0.28 - 0.58)	12 (19.0)	0.88 (0.46 - 1.70)
P			**<0.001**		0.71
Calcium Supplement					
No	568 (79.2)	374 (85.8)	1.00	55 (87.3)	1.00
Yes	149 (20.8)	62 (14.2)	0.63 (0.46 - 0.87)	8 (12.7)	0.55 (0.26 - 1.19)
P			**0.005**		0.13
Alcoholic Drinks, per week					
<7	501 (69.9)	301 (69.0)	1.00	49 (77.8)	1.00
≥7	216 (30.1)	135 (31.0)	1.04 (0.80 - 1.35)	14 (22.2)	0.66 (0.36 - 1.27)
P			0.76		0.19
Fiber Intake, per week					
<21.22	357 (49.8)	156 (35.8)	1.00	21 (33.3)	1.00
≥21.22	360 (50.2)	280 (64.2)	1.76 (1.38 - 2.25)	42 (66.7)	1.98 (1.15 - 3.42)
P			**<0.001**		**0.01**
Hormone Contraception					
Never	131 (44.7)	93 (57.4)	1.00	30 (73.2)	1.00
Ever	162 (55.3)	69 (42.6)	0.60 (0.41 - 0.88)	11 (26.8)	0.30 (0.14 - 0.61)
P			0.10		**0.001**
Hormone Replacement Therapy					
Never	182 (62.1)	115 (71.0)	1.00	36 (87.8)	1.00
Ever	111 (37.9)	47 (29.0)	0.67 (0.44 - 1.01)	5 (12.2)	0.23 (0.09 - 0.60)
P			0.06		**0.003**
Menopause					
Pre-menopausal	38 (13.0)	13 (8.0)	1.00	0 (0)	na
Post-menopausal	255 (87.0)	149 (92.0)	1.71 (0.88 - 3.31)	41 (100)	
P			0.11	-	
Tumour Location					
Distal colon or Rectum	na	277 (66.6)	na	7 (11.5)	na
Proximal colon		139 (33.4)		54 (88.5)	
Microsatellite-instability					
MSS	na	436 (100)	na	36 (57.1)	na
MSI		0 (0)		27 (42.9)	

CRC = colorectal cancer; na = not applicable; OR = odds ratio

[a] OR estimated by multinomial logistic regression model.

The association between diabetes, smoking, NSAIDs and colorectal cancer patients who were stratified by the *BRAF* mutation was investigated with a multinomial logistic regression analysis (**Table 4.5.2**). In univariate analysis, which was adjusted for age and sex, *V600E* colorectal cancer patients exhibited a significant association with diabetes (OR = 2.53; 95% CI, 1.38 – 4.64), current smoking (OR = 2.42; 95% CI, 1.13 – 5.20) and a borderline significant association with former smoking (OR = 1.85; 95% CI, 0.99 – 3.44) and NSAIDs (OR = 0.57; 95% CI, 0.32 – 1.02). The independent effects of diabetes, smoking and NSAIDs were also investigated with a multivariate model, which simultaneously adjusted for age, sex, body mass index, height, education, fiber, calcium supplements, alcohol and colorectal cancer screening. In a multivariate model *V600E* colorectal cancer patients were significantly associated with diabetes (OR = 2.06; 95% CI, 1.09 – 3.88), former (OR = 1.85; 95% CI, 0.99 – 3.44) and current smoking (OR = 2.42; 95% CI, 1.13 – 5.20) and inversely associated with NSAIDs (OR = 0.52; 95% CI, 0.29 – 0.95).

BRAF Wt colorectal cancer patients exhibited a significant association in univariate analyses with diabetes (OR = 1.83; 95% CI, 1.33 – 2.53), former (OR = 1.42; 95% CI, 1.07 – 1.88) and current smoking (OR = 1.80; 95% CI, 1.26 – 2.57). Patients exhibited a borderline significant inverse association with NSAIDs (OR = 0.78; 95% CI, 0.61 – 1.00). In multivariate analysis, *BRAF* Wt colorectal cancer patients were significantly associated with diabetes (OR = 1.61; 95 %CI, 1.15 – 2.26), former smoking (OR = 1.36; 95% CI, 1.01 – 1.82), current smoking (OR = 1.72; 95% CI, 1.18 – 2.52), and inversely associated with NSAIDs (OR = 0.71; 95% CI, 0.54 – 0.92). Although not statistically significant, the magnitude of the point

estimates for the association between diabetes, smoking, NSAIDs, and patients who had a *BRAF* Wt tumour were less than the association with patients who had a *V600E* tumour.

The association between body mass index and colorectal cancer patients stratified by the *V600E* mutation was investigated with a multinomial regression model (**Table 4.5.3**). In men, an elevated BMI two years prior to diagnosis was significantly and positively associated with *BRAF Wt* colorectal cancer (P trend = 0.02), but not with *V600E* colorectal cancer (P trend = 0.38). In women, there was no evidence that an elevated BMI was associated with either *BRAF* Wt (P trend = 0.30) or *V600E* colorectal cancer (P trend = 0.84).

Table 4.5.2 The association between diabetes, smoking, non-steroidal anti-inflammatory drugs and colorectal cancer patients stratified by the *V600E BRAF* mutation status of their tumour.

Exposure	Controls	*BRAF* Wt Tumour			*V600E BRAF* Tumour		
	No. (%)	*No. (%)*	Univariate[a]	Multivariate[b]	*No. (%)*	Univariate[a]	Multivariate[b]
			OR[c] (95% CI)	*OR (95% CI)*		*OR (95% CI)*	*OR (95% CI)*
Diabetes							
No	623 (86.9)	342 (78.4)	1.00	1.00	45 (71.4)	1.00	1.00
Yes	94 (13.1)	94 (21.6)	1.83 (1.33 - 2.53)	1.61 (1.15 - 2.26)	18 (28.6)	2.53 (1.38 - 4.64)	2.06 (1.09 - 3.88)
P			**<0.001**	**0.006**		**0.003**	**0.03**
Smoking							
Never	270 (37.7)	123 (28.2)	1.00	1.00	18 (28.6)	1.00	1.00
Former	342 (47.7)	226 (51.8)	1.42 (1.07 - 1.88)	1.36 (1.01 - 1.82)	32 (50.8)	1.85 (0.99 - 3.44)	1.84 (0.97 - 3.48)
Current	105 (14.6)	87 (20.0)	1.80 (1.26 - 2.57)	1.72 (1.18 - 2.52)	13 (20.6)	2.42 (1.13 - 5.20)	2.35 (1.06 - 5.24)
P-trend			**0.001**	**0.004**		**0.02**	**0.02**
NSAIDs							
No	440 (61.4)	291 (66.7)	1.00	1.00	46 (73.0)	1.00	1.00
Yes	277 (38.6)	145 (33.3)	0.78 (0.61 - 1.00)	0.71 (0.54 - 0.92)	17 (27.0)	0.57 (0.32 - 1.02)	0.52 (0.29 - 0.95)
P			**0.05**	**0.01**		**0.06**	**0.03**

OR = odds ratio; NSAIDs = non steroidal anti-inflammatory drugs

[a] Adjusted for patient age and sex.

[b] Multivariate adjustment for age, sex, body mass index, height, education, fibre intake, calcium supplements, alcohol and colorectal cancer screening.

[c] Odds ratio estimated by multinomial logistic regression model.

Table 4.5.3 The association between body mass index and colorectal cancer patients stratified by sex and the *V600E BRAF* mutation status of their tumour.

Sex	Exposure	Controls	*BRAF* Wt tumour			*V600E BRAF* tumour		
		No. (%)	*No. (%)*	Univariate[a] *OR (95% CI)*	Multivariate[b] *OR (95% CI)*	*No. (%)*	Univariate[a] *OR (95% CI)*	Multivariate[b] *OR (95% CI)*
Men	**BMI (Kg/m²)**							
	≤24.9	109 (25.7)	56 (20.4)	1.00	1.00	3 (13.6)	1.00	1.00
	25 - 29.9	229 (54.0)	133 (48.5)	1.13 (0.77 - 1.66)	1.05 (0.70 - 1.57)	13 (59.1)	2.06 (0.58 - 7.39)	1.68 (0.46 - 6.13)
	≥30	86 (20.3)	85 (31.0)	1.92 (1.24 - 2.99)	1.73 (1.08 - 2.77)	6 (27.3)	2.54 (0.62 - 10.43)	2.00 (0.47 - 8.47)
	P-trend			**0.003**	**0.02**		0.21	0.38
Women	**BMI (Kg/m²)**							
	≤24.9	112 (38.2)	67 (41.4)	1.00	1.00	13 (31.7)	1.00	1.00
	25 - 29.9	116 (39.6)	57 (35.2)	0.82 (0.53 - 1.27)	0.74 (0.46 - 1.20)	19 (46.3)	1.41 (0.67 - 2.99)	1.10 (0.47 - 2.53)
	≥30	65 (22.2)	38 (23.5)	0.98 (0.59 - 1.61)	0.77 (0.43 - 1.35)	9 (22.0)	1.19 (0.48 - 2.94)	0.89 (0.32 -2.47)
	P-trend			0.80	0.30		0.62	0.84

OR = odds ratio; BMI = body mass index

[a] Adjusted for age

[b] Multivariate adjustment for age, diabetes, smoking, non steroidal anti-inflammatory drugs, education, fibre intake, calcium supplement, colorectal cancer screening and height. Odds ratios for women were also adjusted for hormone contraceptive use, horomone replacement therapy and menopausal status.

Chapter 5 – Discussion

5.1 Introduction

The greatest strength of this study is that it takes a multidisciplinary and molecular pathology epidemiological approach to investigating the hereditary basis of colorectal cancer in Newfoundland. Another strength of this research project is that it has recruited a large number of incident and population-based colorectal cancer patients, as well as a large number of population-based controls, which has enabled multivariate statistics to be computed.

Population-based studies offer advantages over selected cohort studies, particularly because they are less susceptible to biases that can affect the validity and generalizibility of study findings. Consequently, the findings of this research project are likely to be highly generalizable to similar patient populations. They are also likely to be generalizable from a molecular genetics perspective, as Newfoundland has the best generalizibility to Caucasian populations when compared to twelve other founder populations [8].

This study also has several limitations that should be noted. First, population-based studies are susceptible to a non-responder bias, particularly if a high proportion of eligible patients are not successfully recruited. The bias arises from the fact that non-responders may be systematically different from responders, which can affect both the validity and generalizibility of findings. That being said, the present study achieved a response rate (64%) that is greater than what has been typically achieved by comparable population-based studies [288, 289]. However, it should be cautioned that the study cohort excluded

patients who were diagnosed at 75 years of age or greater, which may reduce the generalizibility of its findings to the population. Second, this analysis has made numerous comparisons and it is possible that some findings are due to chance. It should also be noted that for some analyses statistical power was limited by small sample size. Third, case-control studies are retrospective in nature and cannot determine causality, but there is considerable evidence to indicate that diabetes, smoking and NSAIDs exert causal effects with respect to colorectal carcinogenesis. Additionally, exposure data was collected retrospectively and was self-reported, which could potentially reduce the internal validity of this study because of recall bias. Fourth, although the participation rate of controls (49%) was relatively high, respondents differed from non-respondents [290]. Lastly, the Newfoundland and Labrador population may not be representative of other populations; however as previously discussed, from a molecular genetics perspective the NL population has the greatest generalizibility to Caucasian populations [8].

5.2 Epidemiology

The microsatellite-instability phenotype is associated with a distinct pathway of colorectal cancer carcinogenesis that occurs in approximately 15% of all tumours. In the present study, 11% of colorectal tumours were found to be MSI-H. The low proportion of MSI-H tumours is likely explained by the fact that MSI-H is positively associated with age and the present study has excluded patients greater than 75 years of age at diagnosis. The microsatellite-instability phenotype is also strongly associated with Lynch syndrome and 28% percent of

all patients who had a MSI-H tumour carried a pathogenic variant of *MLH1*, *MSH2*, *MSH6* or *PMS2*.

The findings from previous studies [199, 209, 210] estimate that between 10% and 18% of colorectal tumours are *V600E* mutation positive. In the current study, the prevalence of the mutation (12%) occurred at the low end of this range, which is likely explained by the fact that the *V600E* mutation is also positively associated with increasing age. The mutation is also strongly associated with MSI-H tumours and it occurred in 50% of MSI-H tumours (n = 38), but only in 8% of MSS tumours (n = 27).

A small proportion of patients (3.0% of the study population) could not be stratified according to the molecular pathology of their tumours (shaded grey in **Figure 2**). The etiology of these 17 patients who had a MSI-*BRAF* Wt tumour is unclear, as these patients could not be linked with either Lynch syndrome or the *V600E* mutation. Eight patients identified as "possible serrated pathway" had a tumour that was hypermethylated at the *MLH1* promoter. As *MLH1* promoter methylation is strongly associated with the serrated pathway, these patients likely share a similar etiology as patients who have a MSI-*V600E* tumour. The 9 other patients, identified as 'possible Lynch syndrome', had MSI-*BRAF* Wt tumors that were unmethylated at the *MLH1* promoter. These tumours are, therefore, unlikely to be associated with epigenetic dysregulation and the serrated neoplasia pathway. However, since these tumours are deficient in at least one MMR protein on IHC, these patients may be carrying a pathogenic MMR variant that was undetected by our methods.

There are two additional possibilities that may be responsible for our inconclusive findings. These molecular pathology results for these patients may be related to a novel mechanism of colorectal carcinogenesis, which results in the MSI-H phenotype without the inactivation of *MLH1*. Alternatively, these findings may be the result of a technical failure or error.

5.3 Inherited Colorectal Cancer

Colorectal cancer is one of the most hereditary of all the common malignancies. In the present study, 32% of patients had an immediate family member affected by colorectal cancer and these patients were suspected of having inherited a predisposition to develop colorectal cancer. Our definition of hereditary colorectal cancer did not include patients who had a second-degree relative affected by colorectal cancer – a known colorectal cancer risk factor [44] – and, therefore, our estimate of hereditary colorectal cancer may be conservative. The findings from twin and population-based studies [11-13] indicate that between 20% and 30% of all colorectal cancer patients are attributable to inherited factors. Therefore, the findings of the present study suggest that the prevalence of inherited colorectal cancer in the Newfoundland population is high. The reasons for this are unclear, but it may be related to greater environmental risk, founder effects in unidentified genes or better ascertainment of familial risk. It may be attributable, in part, to Newfoundland's founder population and a high prevalence of mutations in known susceptibility genes (i.e. causing Lynch syndrome and familial adenomatous polyposis), but a recent analysis by Woods *et al* [291] indicates that this is not the case. Rather,

these findings suggest that the high prevalence of hereditary colorectal cancer in the NL population is likely the result of novel susceptibility factors, either genetic, environmental or both.

5.3.1 High-Risk Patients

Well-defined inherited colorectal cancer syndromes are estimated to account for approximately 5% of all CRC patients, and Lynch syndrome accounts for the majority of these patients [115]. Lynch syndrome was attributable for 3% of patients in this study population. However, approximately 9% of the study population satisfied high-risk criterion and greater than two-thirds of these patients were unrelated to Lynch syndrome and had an unknown etiology. Approximately half of all patients (56%) who satisfy the AC-1 criteria are not affected by Lynch syndrome and have a microsatellite-stable tumour – meeting the criteria for FCCTX. This finding is consistent with other population-based studies [37, 266, 267, 292], which have found 40% of patients who satisfy the Amsterdam I criteria to have a microsatellite-stable tumour.

The etiology of FCCTX is unknown and whether it is caused by highly penetrant gene mutations is unclear. There is, however, accumulating evidence from genetic linkage studies [27-32, 268, 293, 294] indicating that FCCTX is more likely to be caused by a number of low-to-moderately penetrant risk alleles, rather than a single highly penetrant disease allele. In the present study, the estimate of lifetime colorectal cancer risk in FDRs of patients identified as FCCTX (lifetime risk = 39%) is consistent with a highly penetrant monogenic disease. However, after removal of the affected family members who were necessary to

satisfy the FCCTX criteria, the estimate of risk (lifetime risk = 20%) is lower and no longer supportive of a highly penetrant monogenic disease. This finding is consistent with one other study[37] that has investigated the risk of colorectal cancer associated with FCCTX. Lindor *et al* [37] reported that the incidence of colorectal cancer in FCCTX families was significantly less when compared to Lynch syndrome kindreds and that the risk of colorectal cancer in family members of FCCTX patients was only moderately elevated (SIR = 2.7; 95% CI, 1.9 – 3.4). Our estimate of colorectal cancer risk (SIR = 4.9; 95% CI, 2.3 – 9.4) is comparable and provides further support for the argument that FCCTX is unlikely to be caused by a single highly penetrant variant.

FCCTX patients have been recognized to have a clinicopathological profile that is distinct from that of Lynch syndrome patients [37, 265-267, 295]. Consistent with these observations, FCCTX patients were diagnosed at a significantly later age than Lynch syndrome patients ($P = 0.02$) and had a significantly reduced incidence of multiple tumours ($P = 0.04$). In addition, 2 of 15 (13%) tumours from FCCTX patients contained the *V600E BRAF* mutation and thus likely to be associated with the serrated neoplasia pathway, which is supportive of the suggestion that FCCTX represents a heterogenous disease.

The mean age of colorectal cancer diagnosis in FCCTX families has consistently been found to be greater than 50 years of age [37, 265-267]. Therefore, it would appear that the age criterion of the FCCTX criteria could limit the capacity to identify similar patients who have a family history suggestive of autosomal dominant disease. Thus, we introduced the M-FCCTX criteria to remove the age criterion of the FCCTX criteria (colorectal cancer diagnosed <50

years of age), in order to identify additional patients whose family histories suggest dominant inheritance, but who have a later age of onset. An additional 16 (3.3%) patients were identified and the clinicopathological profile of these patients, as well as the cancer-risk in FDRs, was comparable to FCCTX. As expected, age at cancer diagnosis in FDRs was slightly higher than in FCCTX families. However, after the age of 50 years the risk of colorectal cancer in FDRs of FCCTX and M-FCCTX patients was virtually identical. These findings suggest that the current FCCTX criteria lack sensitivity to identify families who exhibit a strong susceptibility to develop colorectal cancer. The FCCTX capture approximately 3% of the study population who have a severe but unknown disease predisposition. However, by eliminating the restrictive age-criterion of the FCCTX criteria, an additional 3% of the study population (i.e. 6% in total) fulfills high-risk family history criteria (i.e. M-FCCTX criteria). Although the number of patients meeting either the FCCTX or M-FCCTX criteria represents only a small proportion of patients, they represent double the number of Lynch syndrome patients.

5.3.2 Intermediate-Risk Patients

The etiology of hereditary colorectal cancer is poorly understood. Although multiple susceptibility loci have been identified, these variants explain little of the excess familial risk. The findings of the present study, suggest that the presence of the *V600E BRAF* mutation and the occurrence of multiple tumours are associated with a 2-fold greater family history of colorectal cancer when compared to families of patients without either of these features.

The *V600E BRAF* mutation is strongly associated with sessile serrated adenomas and appears to be a sensitive and specific molecular marker of the sessile serrated adenoma neoplasia pathway [220]. In the present study, the risk of developing colorectal cancer was significantly elevated for family members of patients who had a *V600E* tumour. Compared to family members of patients who had a solitary *BRAF* Wt tumour, the risk of developing colorectal was 2.7-fold greater in family members of patients who had a *V600E* tumour. Given the strong association between the *V600E* mutation and the SSA pathway, this finding may indicate that SSA pathway is associated with an elevated colorectal cancer risk for family members.

The serrated neoplasia pathway is a relatively new discovery [182] and its etiology is unclear. However, the findings of the current study provide further support for the hypothesis that the serrated pathway has an inherited component [184, 185]. The serrated pathway is linked to two cancer syndromes associated with serrated precursor lesions and colorectal cancer, namely hyperplastic polyposis syndrome and serrated pathway syndrome [185]. The former is a rare polyposis syndrome that is associated with substantial colorectal cancer risk and

appears to be hereditary. The latter was described by Young *et al* [255], who provided evidence for an autosomal dominant colorectal cancer syndrome that is associated with the development of advanced serrated lesions and with the development of MSI-variable, *V600E BRAF*, proximally located tumours. There is also some evidence that clinical and molecular markers linked with the serrated pathway are associated with inherited colorectal cancer. An elevated family history of cancer has been observed in patients who have proximally located serrated polyps [296], a *V600E* tumour [211, 255, 297-299] or a CIMP-positive tumour [199, 300]. However, the evidence is limited as most studies have been small or have used selected patient cohorts. To this author's knowledge, the present study is the largest and most thorough investigation of the association between family history and *V600E* colorectal cancer.

Patients who had *BRAF* Wt multiple tumours also had a significantly elevated family history of colorectal cancer. The incidence of colorectal cancer in family members is approximately 2-fold greater relative to family members of index patients who had a solitary *BRAF* Wt tumour. The explanation for this finding is not immediately apparent. However, it is highly unlikely to be due to known colorectal cancer syndromes (i.e. Lynch syndrome or polyposis syndromes), as these patients were excluded from this particular analysis. It may, however, be explained by recent findings from colonoscopy screening and molecular studies, which suggest that the development of a synchronous or metachronous colorectal tumour may be associated with the serrated pathway. For example, the presence of a large or proximally located serrated polyp is now recognized to be a strong risk factor for the development of a synchronous or

metachronous tumour [296, 301, 302] and one study has suggested that a large or proximally located serrated polyp is a greater risk factor than the presence of multiple tubular adenomas. Molecular studies [303-305] are also reporting that the development of multiple colorectal tumours is strongly associated with features of the serrated pathway, such as aberrant DNA methylation, the *V600E* *BRAF* mutation and microsatellite-instability. Additionally, it appears that the traditional serrated neoplasia pathway, which is characterized by neoplasia of the distal colon [306, 307], alterations of *KRAS* rather than *BRAF* and with methylation of *MGMT* [303, 305] are strongly associated with the development of synchronous and metachronous colorectal neoplasia. Unlike the association between family history and patients who had a *V600E* tumor, the elevated familial predisposition observed with patients who had *BRAF* Wt multiple tumours may be attributable to the traditional serrated adenoma neoplasia pathway.

Patients whose tumors contained the *V600E* mutation had a distinct clinical phenotype relative to patients who had a *BRAF* Wt tumour. Patients with a *V600E* tumour were significantly more likely to be female and to have a proximally located tumour. Colorectal tumours that were *V600E* were also associated with a distinct histological phenotype relative to *BRAF* Wt tumours. *V600E* tumours were significantly more likely to be poorly differentiated, to contain tumour-infiltrating lymphocytes, and to have a mucinous component. These findings are consistent with several studies [183] that have reported *V600E* colorectal cancer to be associated with a distinct clinical, molecular, and

histological phenotype. Conversely, a synchronous or metachronous tumour was not associated with any distinct clinical or histological feature.

5.4 V600E Colorectal Cancer

The *V600E BRAF* mutation is an early molecular event in the sessile serrated adenoma pathway of carcinogenesis and it is unclear if later events, such as epigenetic inactivation of *MLH1* and the microsatellite-instability phenotype, influence the clinicopathologocal phenotype and elevated familial cancer-risk observed in patients who have a *V600E* tumour. The clinicopathological phenotype and cancer-risk associated with patients who have a *V600E* tumour was investigated according to the microsatellite-instability status of these patient tumours.

The findings suggest that *V600E* colorectal cancer is significantly associated with an elevated risk of colorectal cancer in family members irrespective of tumour microsatellite-instability status. The risk of developing colorectal cancer was significantly elevated in family members of patients who had either a MSS (HR = 2.19) or a MSI (HR = 1.75) *V600E* tumour. These findings suggest that early molecular events in the sessile serrated adenoma pathway of colorectal carcinogenesis may be linked with an elevated familial risk.

Several studies [211, 255, 308] have investigated the association between patients with a *V600E* tumour and a family history of cancer, but only one study [211] has used a population-based approach. A correlation between a family history of colorectal cancer and patients with a MSI-*V600E BRAF* tumour has been previously reported [308]. However, that particular study [308] used a

selected cohort of familial colorectal patients, and only eight MSI tumours were evaluated. A population-based study conducted by Samowitz *et al* [211], reported finding a significant association between patients who had a MSS-*V600E BRAF* tumour and a family history of colorectal cancer (OR = 4.23; 95% CI, 1.65 – 10.84). However, no significant association was found amongst patients who had a MSI-H-*V600E* tumour (OR = 0.64; 95% CI, 0.18 – 2.19). The findings of the present study are the first to report an association between patients who have a MSI-*V600E* tumour and a family history of colorectal cancer in an unselected series of population-based patients.

It has been postulated [185] that the burden of colorectal cancer arising from the serrated pathway could be explained by a co-dominant model. In a co-dominant model of inheritance, the more severe phenotype associated with HPS may be the result of a carrier of two mutated alleles of the hypothesized gene. The increased predisposition to develop sessile serrated adenomas and serrated pathway neoplasia may be attributable to carriers of one mutated allele.

The present study investigated the mode of disease inheritance associated with the development of *V600E* colorectal cancer by comparing the risk of developing colorectal cancer in siblings and parents. A far greater incidence of colorectal cancer in siblings compared to parents would be expected if inherited factors were transmitted in an autosomal recessive manner. Alternatively, if the mode of disease inheritance were multifactorial or autosomal dominant, one would expect that the incidence of colorectal cancer to be comparable between parents and siblings. This analysis has made the assumption that inherited variants play a role in the etiology of *V600E* colorectal cancer, which may not be

true. Nevertheless, the findings of the present study provide some evidence for different modes of inheritance depending on the MSI status of the index patient's tumour. In patients who have a MSS-*V600E BRAF* tumour, the incidence of colorectal cancer was significantly greater in siblings than in parents; whereas the incidence was similar between parents and siblings for patients who had a MSI-*V600E* tumour. These findings may suggest that MSS-*V600E BRAF* colorectal cancer disease susceptibility is associated with recessively inherited factors; whereas the incidence of colorectal cancer in families of patients who have a MSI-*V600E BRAF* tumour was more suggestive of dominant or multi-factorial inheritance.

We investigated the incidence of extra-colonic tumours in family members of index patients. Family members of index patients who had a *V600E* tumour had a significantly elevated incidence of non-melanoma skin cancer compared to family members of patients who had a *BRAF* Wt tumor. Interestingly, the results of a recent prospective study [309] found a 2-fold increase in the risk of colorectal cancer following a diagnosis of non-melanoma skin cancer. Colorectal and non-melanoma skin cancer share some of the same risk factors, such as smoking [246]. Furthermore, susceptibility to develop both non-melanoma skin cancer and *V600E* colorectal cancer appears to be modified by the interaction between environmental exposures and polymorphisms in the base excision repair genes, *XRCC1* and *OGG1*, respectively [248, 310]. A recent case-control study [248] reported that ever-smokers who were homozygous for the *OGG1 (S326C)* polymorphism were twice as likely to have a *V600E* tumour. The observed association between *V600E* colorectal cancer and non-melanoma skin cancer

observed in the present study might be explained by an inherited variant that would increase susceptibility to both types of cancer by attenuating DNA repair capability.

The clinicopathological phenotype of patients who had a *V600E* tumour was found to be dependent on microsatellite-instability status. Regardless of MSI status, patients with a *V600E* tumour were significantly more likely to be female and to have a proximal tumour than patients who had a *BRAF Wt* tumour. However, patients with a MSI tumour, but not those who had a MSS tumour, were significantly older at diagnosis. This finding is consistent with the MSI phenotype tending to occur in tumors diagnosed later in life. Additionally, patients who had a MSI tumour were more likely to have multiple tumours. These findings provide further evidence that *V600E* colorectal cancer is associated with a distinct clinical, molecular and pathological phenotype [208, 211, 219].

The *V600E* mutation in microsatellite-stable colorectal cancer has been identified [211] as a marker for poor prognosis. In the present study, patients who had a MSS-*V600E* tumour were significantly more likely to be diagnosed at a later disease stage compared to patients who had a *BRAF Wt* tumour. However, after multivariate adjustment, this result was no longer statistically significant. Microsatellite-instability is recognized as a positive prognostic marker and patients who had a MSI-*V600E* tumor were more likely to be diagnosed at an early stage of disease compared to patients who had a MSS-*BRAF Wt* tumour. However, this result did not reach statistical significance in multivariate analysis.

Until recently there has been insufficient data to make evidence-based decisions regarding best screening and surveillance for serrated lesions. It is now evident [296, 301, 302] that serrated polyps, particularly large and proximally located lesions, harbour substantial malignant potential. It is also now recognized that the serrated pathway may be associated with 20% of colorectal cancers and with 30% of interval colorectal cancers [183]. These revelations have spurred recommendation for surveillance guidelines to recognize the importance and malignant potential of serrated polyps [311]. The findings of the current study may also be relevant to future colorectal cancer screening practices. Risk-stratification is an important facet of population-based colorectal cancer screening guidelines, which utilize family history information to identify those at increased risk of colorectal cancer and to provide them with earlier and more aggressive screening. In the current study, family members of colorectal cancer patients who had either a *V600E* or *BRAF Wt* multiple tumours were twice as likely to develop colorectal cancer. These findings suggest that markers of the serrated pathway could be useful as markers of increased cancer risk in family members. Although this study is the largest and most thorough investigation of hereditary colorectal cancer and markers of the serrated pathway to date, its findings will need to be validated by additional studies. Nevertheless, the findings suggest that markers of the serrated pathway could be utilized to identify families who are at increased risk of developing colorectal cancer and who should be enrolled in high-risk screening programs.

5.5 Epidemiological Factors and V600E Colorectal Cancer

The determinants and consequences of insulin resistance syndrome are established colorectal cancer risk factors [61], but it is unclear if these risk factors are equally involved with alternative pathways of colorectal carcinogenesis. The *V600E BRAF* mutation in colorectal cancer has been linked to a number of lifestyle and dietary factors, including a low-fiber diet [237], smoking [237, 245-248] and NSAIDs [237], but the association between *V600E* colorectal cancer and diabetes has not been investigated.

The findings of this analysis indicate that diabetes and smoking are strongly associated with the risk of developing *V600E* colorectal cancer. Additionally, the regular use of NSAIDs is associated with a significantly diminished risk of developing *V600E* colorectal cancer. These findings suggest that metabolic derangement and inflammatory mechanisms may play an important role in the etiology of *V600E* colorectal cancer and the sessile serrated adenoma pathway of carcinogenesis.

Similar to colorectal carcinogenesis, insulin resistance syndrome is a complex and heterogenous disease that has numerous exogenous and endogenous risk factors. Its pathology is believed to involve impaired insulin signaling through the PIK3 pathway, but with intact insulin signaling via the MAPK pathway, which results in impaired glucose uptake and systemic inflammation [55]. These abnormalities are also implicated with the pathology of cardiovascular disease, diabetes and cancer.

Diabetes is a clinical end-point of long standing cellular insulin resistance and is strongly associated with an increased risk of developing colorectal cancer [61].

A recent meta-analysis suggests that diabetes increases the risk of developing colorectal cancer by 30% (95% CI, 20% - 40%) [63]. In the present study, diabetes was significantly associated with the development of *V600E* colorectal cancer. This is the first study to report that diabetes or any metabolic abnormalities linked with insulin resistance syndrome is associated with the development of *V600E* colorectal cancer. That being said, this finding is supported by evidence indicating that metabolic derangement may be associated with serrated pathway neoplasia. For example, *V600E* colorectal cancer is recognized to be strongly associated with the proximal tumour location [209] and diabetes has consistently been linked [58, 59, 312-314] as a risk factor for neoplasia of the proximal colon, but not for the distal colon. One study [314], which prospectively investigated the risk of developing colorectal cancer in a cohort of post-menopausal diabetics, is particularly relevant since the *V600E BRAF* colorectal cancer is strongly associated with older women who are post-menopausal. In that study, the risk of colorectal cancer after fourteen years of observation was significantly elevated for the proximal colon (RR = 1.9; 95% CI, 1.3 – 2.6), but not for the distal colon (RR = 1.1; 95% CI, 0.6 – 1.8) or for the rectum (RR = 0.8; 95% CI, 0.4 – 1.6).

There is also evidence suggesting that serrated pathway precursor lesions are linked with biochemical and clinical abnormalities that are associated with insulin resistance syndrome. First, the occurrence of hyperplastic polyps — the precursor lesions of the serrated pathway — are associated with elevated serum concentrations of insulin and insulin-like growth factor-1 (IGF-1). In a cohort of consecutively ascertained colonoscopy patients, Yoshida *et al* [239] found elevated serum insulin concentrations to be positively correlated with the

presence of hyperplastic polyps. Moreover, the correlation was found to be strongest for right-sided hyperplastic polyps, which are the polyps that are most likely to harbor the *V600E BRAF* mutation. Second, it has also been reported [240, 241] that acromegaly, a condition characterized by elevated plasma IGF-1, is associated with the development of hyperplastic polyps and colorectal cancer. Furthermore, the risk of developing hyperplastic polyps in these patients is positively correlated with insulin resistance, insulin, and IGF-1. Third, a recent study [238] has reported that adiposity in women, which is a strong determinant of insulin resistance and diabetes, is positively associated with the incidence of hyperplastic polyps. These findings, although not definitive, provide some evidence that metabolic derangement may be associated with serrated pathway neoplasia and are supportive of this study's findings.

Chronic inflammation is linked with the underlying pathophysiology of insulin resistance [315], diabetes [316] and colorectal cancer [78]. It is for this reason that the regular use of NSAIDs is associated with a reduction in the risk of developing diabetes [317] and colorectal cancer [80] via its inhibitory effects on inflammation. In the current study, the regular use of NSAIDs was inversely associated with the development of *V600E* colorectal cancer. The association between *V600E* colorectal cancer and NSAIDs has been investigated in only one other study [237], which reported a borderline significant inverse association (OR = 0.7; 95% CI, 0.5 – 1.0). Nevertheless, several other studies have found the regular use of NSAIDs to be inversely associated with the development of advanced right-sided serrated lesions [236] and hyperplastic polyps [72, 233]. The findings of the present study suggest that inflammatory mechanisms may

play a particularly important role in serrated pathway neoplasia. These findings are also consistent with the hypothesis [197] that chronic inflammation is the pathogenic mechanism causing dysregulation of gene promoter methylation and the serrated pathway of carcinogenesis.

Despite the proven efficacy of NSAIDs as a colorectal cancer chemopreventative, the United States Preventative Task Force [79, 80] does not support their regular use, arguing that the benefits do not outweigh the harms. It could be argued, however, that for the appropriate at-risk population the chemopreventative benefits of NSAIDs could potentially outweigh the harms. The findings of the present study raise the possibility that those predisposed to serrated pathway neoplasia could benefit from the chemopreventative use of NSAIDs. Furthermore, as the use of salicylates therapy is also linked with improved glucose metabolism [318], insulin sensitivity [319] and a reduced risk of diabetes [317] the rationale for intervention with anti-inflammatory drugs in these particular patients may be strengthened.

This analysis provides further evidence that smoking is strongly associated with patients who have a *V600E* tumour. After multivariate adjustment, current smoking was a strong and independent risk factor for *V600E* colorectal cancer (OR = 2.35; 95% CI, 1.06 – 5.24). A substantial body of evidence [249] has identified smoking as a risk factor for colorectal cancer. Recently, however, the association between smoking and colorectal cancer has been recognized to be greatest for those with hyperplastic polyps [242, 243] and for patients who have a microsatellite-instable [244, 245], CIMP or *V600E* tumour [237, 245-248]. The reason for the strong association is unclear, but it may be related to a link found

[76, 77] between smoking and increased CpG island methylation. The strength and consistency of the association found between these molecular alterations and smoking provides support for the hypothesis that smoking plays a role in the etiology of the serrated pathway of carcinogenesis. In light of the findings of the current study, which suggest that the *V600E* mutation is strongly associated with metabolic derangement and inflammation, this author is inclined to posit that the strong relationship that has been observed between smoking and the *V600E* *BRAF* mutation may be attributable, in part, to the effect of smoking in attenuating insulin sensitivity [320, 321], IGF-1 [322], and increasing inflammation [323].

Adiposity is associated with an increased risk of colorectal cancer via its adverse effects on insulin sensitivity, inflammation, growth factors, and steroid hormones [64, 65]. Although one study [238] has recently linked adiposity with the development of hyperplastic polyps, the present study found no evidence for an association between an elevated BMI and the development of *V600E* colorectal cancer in either men (P trend = 0.36) or women (P trend = 0.84). However, in patients with a *BRAF* Wt tumour, an elevated BMI was found to be a significant risk factor for men (P trend = 0.02), but not for women (P trend = 0.30). The observed disparity between men and women is consistent with the findings from a recent meta-analysis [66], for which a number of mechanisms have been proposed [64, 65].

The findings of this case-control study may provide some insight into the unknown etiological basis of *V600E* colorectal cancer and the serrated pathway of carcinogenesis. The findings suggest that the etiology of the serrated neoplasia

pathway and *V600E* colorectal cancer may be involved with metabolic and inflammatory mechanisms. Reports from other epidemiological studies would also support this hypothesis, as serrated pathway neoplasia has been linked to environmental, dietary and biochemical factors that are associated with insulin resistance syndrome, including adiposity, smoking, diabetes, low-fiber diet, NSAIDs, insulin resistance, elevated insulin and IGF-1. There is also support for this hypothesis from a genetic association study [324], which discovered serrated pathway colorectal cancer patients (i.e. CIMP or MSI-H tumours) to be associated with gene variants that are implicated with insulin signaling and inflammatory pathways. These findings suggest that the etiological basis of the serrated pathway may be associated with environmental, lifestyle and genetic factors that converge on insulin signaling and inflammatory pathways. Additionally, the findings of this case-control analysis may be applicable to future colorectal cancer screening, surveillance and prevention practices, as they suggest that individuals who are diabetic or who smoke are particularly at-risk for developing serrated pathway neoplasia.

Chapter 6 – Summary of Findings

This research project has taken a transdisciplinary and molecular pathology epidemiology approach to investigating the etiology of hereditary colorectal cancer. The greatest strengths of this study are its molecular pathology approach and the successful recruitment of a large number of population-based patients and controls.

The findings of this research project indicate that only a small proportion of patients are affected by Lynch syndrome (3%). However, there are additional patients who fulfill high-risk family history criteria (i.e. Amsterdam I criteria), but have an unknown etiology. The risk of colorectal cancer in family members of these patients (i.e. FCCTX) is substantial; however, the risk is not suggestive of being caused by a highly penetrant autosomal dominant disease allele. Additionally, the findings of this research suggest that the age criterion of the FCCTX criteria is overly restrictive. By eliminating the age criterion of the FCCTX criteria additional families are identified who exhibit a similar cancer-risk profile as families identified as FCCTX.

In addition to high-risk patients, 26% of non high-risk patients have at least one FDR affected by colorectal cancer. These intermediate-risk patients are significantly associated with either a *V600E* tumour or a multiple tumour, when compared to low-risk patients. For patients who had either a *V600E BRAF* tumour or a multiple tumour the incidence of colorectal cancer in family members was approximately 2-fold greater compared to patients who had neither of these features. As both of these features are strongly linked with the

serrated pathway of carcinogenesis it provides support for the hypothesis that the serrated pathway has a genetic component. However, our findings also suggest that environmental factors play a role in the etiology of *V600E* colorectal cancer and the sessile serrated pathway of carcinogenesis. The development of *V600E* colorectal cancer was found to be significantly associated with diabetes and smoking, and inversely associated with the use of NSAIDs. Thus, these findings suggest that the molecular etiology and colorectal cancer susceptibility associated with the serrated pathway may be linked with endogenous and exogenous risk factors that affect insulin signaling and inflammatory pathways.

These findings of this research may have relevance and applications to future colorectal cancer screening and prevention practices. Clinical and molecular features of the serrated pathway of carcinogenesis may be useful markers of increased familial risk, which may aid to identify families who should be enrolled in high-risk colorectal cancer screening. Additionally, the findings suggest that those who are metabolically unfit and or who smoke may be particularly at-risk for developing serrated pathway neoplasia.

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