EVALUATION OF A NOVEL, SERUM-BASED BIOMARKER SCREENING TEST FOR COLORECTAL CANCER.

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By

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Abstract

Background: This study evaluates a new serum-based biomarker for colorectal cancer (CRC) screening and diagnosis. The biomarker (GTA-446) is a member of hydroxy -polyunsaturated ultra-long chain fatty acids and was found to be reduced in CRC patients compared to CRC-free subjects. Diagnostic test performance characteristics were used to identify the effectiveness of the test.

Methods: Serum levels of GTA-446 were measured in 4924 subjects who underwent colonoscopy for any reason, pathology results and clinical data were also collected. Two sets of age-matched control subjects were used; First were the lab controls (number=383) which were serum samples collected from Saskatchewan Disease Control Laboratory along with age and gender data. Second, were the endoscopy controls (number=762) which were obtained from the colonoscopy population after being determined to be cancer-free. Cut-off values were calculated using Receiver Operating Characteristic (ROC) curve.

Results: Serum GTA-446 was found to be reduced in 87% of CRC patients. Compared to lab controls, the GTA-446 biomarker has a sensitivity of 87%, specificity of 75%, positive likelihood ratio of 3.6, and negative likelihood ratio of 0.16. Using endoscopy controls to calculate test performance characteristics, the biomarker has a sensitivity of 87%, specificity of 50%, positive likelihood ratio of 1.74, and negative likelihood ratio of 0.24. Also, the level of GTA-446 was found to significantly decline with age ($r=-0.20$, $p<0.01$).

Conclusion: Serum GTA-446 is a potential biomarker for minimally invasive detection of colorectal cancer that compares favorably to other serum-based biomarkers.

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List of Abbreviations

AA	Arachidonic Acid
AGEs	Advanced Glycation End Products
APC	Adenomatous Polyposis Coli
ATBC	Alpha-Tocopherol, B-Carotene Cancer Prevention Study
AUC	Area Under the Curve
BH4	Tetra Hydrobiopterin
BMI	Body Mass Index
CAE	Cholic Acid Equivalent
CARET	Beta-Carotene and Retinol Efficacy Trial
CD	Crohn's Disease
CFAs	Conjugated Fatty Acids
CHO	Carbohydrate
CI	Confidence Interval
CI	Confidence Interval
CIMP+	CPG Island Methylation Phenotype
CIN	Chromosomal Instability
COX	Cyclo-oxygenase
CRC	Colorectal Cancer
CT	Computed Tomography
CYP	Cytochrome P
DCC	Deleted in Colon Cancer
DM	Diabetes Mellitus
DOR	Diagnostic Odds Ratio
EPIC	The European Prospective Investigationof Cancer and Nutrition
Eq	equivalent
ER	Estrogen Receptor
ESI	Electrospray Ionization
FAD	Flavin Adenine Dinucleotide
FAP	Familiial Adenomatous Polyposis
FIT	Fecal Immunochemical Test
FMN	Flavin Mononucleotide
FN	False Negative
FOBT	Fecal Occult Blood Test
FP	False Positive
FPR	False Positive Rate
FSIG	Flexible Sigmoidoscopy
FTICR-MS	Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
GCI	Genomics collaborative Inc.
G-FOBT	guaiac-Fecal Occult Blood Test
GTA	Gamma Tocoenoic Acid
GTA	Gastric Tumour Acids
H2O2	Hydrogen Peroxide
HATs	Histone Acetyl Transferases
HCA	Heterocyclic Amines
HMLH	humann Mut L Homolog 1
HMTs	Histone Lysine Methyltransferases

HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HOCL	Hypochlorous Acid
HR	Hazards Ratio
IBD	Inflammatory Bowel Disease
I-FOBT	immunochemical-Fecal Occult Blood Test
IGF	Insulin Growth Factor
IGF-BP	Insulin Growth Factor-Binding Protein
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
JP	Juvenile Polyposis
LM	Lipid Mediator
LOH	Loss of Heterozygosity
LOX	Lipo-oxygenase
LS	Lynch Syndrome
M/Z	Mass to Charge Ratio
MAP	MUTYH-Associated Adenomatous Polyposis
MCC	Mutated in Colorectal Cancer
MDA	Malondialdehyde
MMR	Mismatch Repair
MPO	Myeloperoxidase
MRM	Multiple Reaction Monitoring
MSI	Microsatelite Instability
MSI-H	Microsatelite instability-High
MSI-L	Microsatelite Instability-Low
MSS	Microsatelite Stable
MTHFR	Methylene Tetra Hydro folate Reductase
MUFAs	Monounsaturated Fatty Acids
MW	Molecular Weight
N	Number
NEFA	Non Esterified Fatty Acid
NF-KB	Nuclear Factor-Kappa B
NHANES	National Health and Nutrition Examination Survey
NO	Nitric Oxide
NOC	N-nitroso compounds
NPV	Negative Predictive Value
ODC	Ornithine Decarboxylase
OR	Odds Ratio
PCA	Polycyclic Aromatic Hydrocarbons
PDI	Phenomenome Discoveries Inc.
PEB	Positive Energy Balance
PJS	Peutz-Jeghers Syndrome
PPARs	Peroxisome Proliferator Activated Receptors
PPV	Positive Predictive Value
pRB	Retinoblastoma Protein
PUFAs	Polyunsaturated Fatty Acids
RER	Replication Error
RNI	Reactive Nitrogen Intermediates
ROC	Receiver operating characteristic
ROI	Reactive Oxygen Intermediates

ROS	Reactive Oxygen Species
RR	Relative Risk
SDCL	Saskatchewan Disease Control laboratory
SFAs	Saturated Fatty Acids
SSS	System Suitability Standard
TGF-B	Tumour Growth Factor-b
TN	True Negative
TNF-α	Tumour Necrosis Factor alpha
TP	True Positive
UC	Ulcerative Colitis
VDR	Vit. D Receptor

1 Epidemiology and Pathogenesis:

Colorectal cancer is the third most common cancer in Canada with age standardized incidence rate of 61 per 100 000 males and 40 per 100 000 females and is the second leading cause of cancer death in Canada. Despite improvements in surgical and chemotherapeutic treatments, colorectal cancer has a poor 5-y survival rate of 63% for males and 64% for females (1). Identifying modifiable factors associated with colorectal cancer is of importance, the ultimate goal being primary prevention (2).

Approximately 95% of the malignant colorectal tumors are adenocarcinomas with the majority arising from adenomas. It takes several years for an adenoma to progress to a carcinoma. The progression to cancer results from the accumulation of multiple genetic changes. Not all adenomas progress to cancer. In fact, the vast majority of adenomas do not progress to carcinoma. The malignant potential of the adenomas has been correlated to their size, histopathology, and degree of dysplasia (3).

2 Acquired Causes Of Colorectal Cancer:

The risk of developing colorectal cancer (CRC) is influenced by both environmental and genetic factors. Acquired risk factors include the following categories: dietary factors, lifestyle factors, side-effects of medical interventions, and comorbid medical conditions (4).

2.1 Dietary Factors

The proportion of colorectal cancers attributed to dietary factors has been estimated to be about 50% (5). Further, approximately 66-77% of colorectal cancers have been suggested to be preventable by an appropriate combination of diet and physical activity (6).

2.1.1 Consumption of Fat and colorectal cancer

The association between total dietary fat and risk of colorectal cancer has been evaluated in numerous epidemiologic studies. Results from these analytic studies have generally been mixed. Whereas some studies have reported positive associations, several studies have observed null and inverse associations. In a pooled analysis of data from 13 case control studies, no evidence of risk to CRC was found after adjustment for total energy intake (7). Moreover, results for total dietary fat across several prospective cohort studies have not been supportive of a significant positive association with CRC (8), although a statistically significant 2-fold association was found in an analysis of women in the Nurses' Health Study (9).

It is becoming apparent that the type of fat should be considered, as well as total fat intake. Total fat consists of different fatty acid families, e.g., saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), n-3 and n-6 polyunsaturated fatty acids (PUFAs) and conjugated fatty acids (CFAs). Plant fats have higher concentrations of

unsaturated fatty acids and tend to be oils, whereas animal fats consist of larger amounts of saturated fatty acids as well as unsaturated fats (10).

Experimental studies have confirmed beneficial and harmful effects of fatty acids on abnormal cell proliferation in colon cancer. In colon cancer, animal experiments and cell culture studies have shown that n-3 PUFAs and CFAs were beneficial. While n-6 PUFAs were harmful; SFAs were ineffective, and MUFA action was inconclusive. In particular, the dietary doses required for tumor suppression by CFAs were strikingly low (<1%) in comparison to the doses required to achieve tumor suppression with n-3PUFAs (5-10%) (11;12). However, few epidemiological human studies have investigated the relationship between CFA intake or tissue CFA concentrations and tumor incidence. A prospective study investigating the effect of conjugated linoleic acid intake as estimated by food questionnaire on the incidence of breast cancer showed no protective effect on cancer development (13).

The confirmed effect of n-6 and n-3 PUFAs on colorectal cancer development in experimental studies was not reproduced in human studies, especially those using food frequency questionnaires to assess PUFAs intake. Also, these studies did not adjust for other confounders that modify and oppose the effect of individual fatty acids. Such modifiers could be luminal e.g., Ca and fiber that interfere with the action of fatty acids on colon cells or genetic e.g., genes responsible for repair of damage induced by fatty acids (7).

In humans, the amount and rate of intake of specific fatty acids are hard to measure. In experimental animals the exact amount of purified fatty acid consumed can be monitored over a desired period. In contrast, humans consume many different kinds of

fatty acids in their diet, a mixture of which may exert opposite effects and contain highly variable amounts of crude fats with unknown purity. A recent large case-control study tried to minimize these problems, and found a moderately strong inverse and dose dependent association in multivariate logistic regression models between colorectal cancer risk and intake of n-3 PUFAs and its main compounds (14).

Recently, a study based its assessment on validated plasma markers for fatty acids such as the concentration of n-3 PUFAs in erythrocyte membrane (15). These studies confirmed that higher levels of n-3 PUFAs are associated with lower cancer risk. These results were confirmed by the recently published huge European Prospective Investigation into cancer and nutrition (N = 478,040 men and women) and the U.S.-based Physicians Health Study (N = 22,071 men) (16).

Generally, the proposed mechanisms by which n-3 PUFAs and n-6 PUFAs affect carcinogenesis are that n-6 fatty acids and their derivatives promote the production of pro-inflammatory eicosanoids, whereas n-3 fatty acids suppress this action (17). PUFAs are the basic constituents of membrane phospholipids and the production of eicosanoids begins with the liberation of PUFA from membrane phospholipids. The major PUFA in the cell membrane is Arachidonic acid [(AA; 20 carbons:4 double bonds, location of first double bond at C6 from the methyl terminal (AA, 20:4n-6)], and both n-3 PUFAs and CFA may compete with AA to block its incorporation into membrane phospholipids or to inhibit the cyclooxygenase(COX) and/or the lipooxygenase(LOX) pathway, leading to reduction in AA-derived eicosanoids. COX and LOX inhibitors efficiently block cell proliferation and induce apoptosis in colon cancer cells (10).

2.1.2 Fruits and vegetables:

Fruits and vegetable intake have been suggested to be associated with reduced risk of many cancers. Several mechanisms have been hypothesized. The constituents of fruits and vegetables, including fiber, micronutrients (such as carotenoids, phenolics, isoflavonoids, isothiocyanates and indoles) demonstrate a range of physiological properties, including anticarcinogenic effects. In particular, the phytochemicals have been reported to induce detoxification enzymes, scavenge free radicals, alleviate inflammation, inhibit malignant transformation, stimulate immune functions and regulate the growth of cancer cells (18;19).

Associations between the consumption of fruit and vegetables and CRC risk have been the focus of a large number of case-control and cohort studies. Nevertheless, a recent review by an international panel of experts concluded that the evidence for an inverse association between CRC risk and higher fruit and vegetables is limited; therefore, further studies are warranted. Discrepancies in the cumulative results to date may be explained by differences in study design and the susceptibility of case-control studies to recall and selection biases (20).

A large prospective collaborative project carried out in 10 different European countries: The European Prospective Investigation of Cancer and nutrition (EPIC) study; investigated how the consumption of total fruit and vegetables is related to CRC risk. The study included 452,755 subjects who completed a dietary questionnaire and were followed up for an average of 8.8 years. They found that consumption of fruits and vegetables was inversely associated with CRC, however this association is moderate (HR:0.86 for the highest vs the lowest quintile) (21).

It was hypothesized that not all fruits and vegetables show the ability to suppress carcinogenesis, and there may be specific subtypes of fruits and vegetables that exhibit anticarcinogenic effects. Recently, cruciferous vegetables, rather than vegetables as a group, have drawn a great deal of attention in cancer research because of their potential protective properties. Cruciferous vegetables, including broccoli, cabbage and cauliflower have been identified as rich sources of carotenoids, vitamin C, folate and soluble fiber, which may play an important role in cancer prevention. In addition, the ability of cruciferous vegetables to protect against neoplastic diseases has been attributed to their high glucosinolate content. Glucosinolates are converted by myrosinase in plant cells and microflora in the gastrointestinal tract to indole-carbinol and isothiocyanate, two phytochemicals that exhibit anticarcinogenic effects in models of animal cancer (22).

In a multi-centre Japanese study, inverse associations were found in the group with the highest consumption of broccoli (OR 0.18) for the risk of colorectal cancer (23). In more recent studies the associations have been less consistent. In the case control studies, a significant inverse association was detected (OR 0.73), whereas in the cohort studies, no overall association with cruciferous vegetable intake was detected (OR 0.96). From the currently available data, it cannot be definitively concluded that the consumption of cruciferous vegetables is associated with the overall risk of colorectal cancer (24).

Another approach to investigating the role of fruits and vegetables in risk of CRC is through an examination of dietary patterns rather than focusing on a single diet, because isolating single nutritional agents from diet is impossible. Dietary pattern

analysis reflects both nutrient/food group intakes and the types of foods that tend to be consumed together in the usual diet and may therefore provide additional insights into the diet and colon cancer relationship in a number of ways; 1) it takes into account the combined effects of foods, 2) there are likely racial/ethnic differences in dietary patterns that may contribute to variations in risk, 3) humans consume meals that include a variety of foods and not individual nutrients, and 4) patterns are more amenable to translation into dietary recommendations (25).

A cohort study was conducted to assess the usual dietary intake of nearly 300,000 men and 200,000 women by use of food frequency questionnaires. The individuals were then followed for up to 5 years to determine association of diet patterns with incidence of colorectal cancer. The authors applied a statistical technique called cluster analysis to identify groups of individuals with similar dietary patterns. Clusters of individuals were identified who ate higher quantities of fruit and vegetables, or diet foods and lean meats, or fatty meats. The largest cluster in both men and women was termed the "many foods" cluster, which contained a suboptimal profile of nutritional intake—this cluster served as the reference group. After controlling for other colorectal cancer risk factors, such as BMI, physical activity and smoking, the authors found that men in the "fruit and vegetable" cluster (characterized by a high intake of fruit and vegetables and a low intake of red meat), had a statistically significant 15% lower risk of colorectal cancer than individuals in the "many foods" cluster. Women in the "fruit and vegetable" cluster had a non-statistically significant 10% reduction in colorectal cancer risk compared with those in the "many foods" cluster (26). Another case control study conducted score analysis on three different dietary patterns; "Western-Southern", "fruit-vegetable", and

"metropolitan". The "fruit-vegetable" pattern was significantly inversely associated with colon cancer risk compared to "Western-Southern" pattern (OR 0.4) followed by "metropolitan" pattern (27).

However, most studies conducted are based on a food frequency questionnaire which is prone to measurement and recall bias as cases may recall dietary exposures differently from controls because of their illness in case control studies (28).

2.1.3 Low fiber intake:

Dietary fibers are polysaccharides that escape digestion in the small intestine. They are further classified according to their solubility, plant source and degree of bacterial fermentation upon reaching the colon (29).

Fiber intake was thought to protect against CRC through speeding up transit, increasing stool bulk, thereby diluting the carcinogens present in the gut lumen and reducing exposure to toxins and adsorption of bile acids (30-32). Several studies conducted found the evidence for dietary fiber to be inconsistent. This could be in part due to dietary measurement methods, source, and chemical composition of the fiber (10;33;33;34).

A study conducted comparing the intake of starch, non-starch polysaccharides and fat among 12 populations worldwide found a strong inverse relationship between starch consumption and large bowel cancer (RR= 0.7) (35). Another theory proposed that starch that escapes digestion and absorption in the small intestine undergoes fermentation by colonic bacteria and thus produces short chain fatty acids such as butyrate which may have a role in reducing the malignant changes of colonic epithelial cells. This was partly

supported by a recent in vitro study that found butyrate and carnitine to inhibit human colon carcinoma cell proliferation and induce apoptosis in human colon carcinoma cells (36).

This attracted more attention to the role of gut flora in producing short chain fatty acids through fermentation and their essential role in maintaining large intestinal health. These findings caused Bolin TD to raise the question whether subclinical malabsorption of CHO (carbohydrates) in Asia and Africa that leads to an increased CHO load and fermentation in the large intestine is the reason behind the lower incidence of CRC compared to western countries (37).

2.1.4 Phytoestrogen Intake

Lignan and isoflavones are dietary phytoestrogens found in plant foods. Lignans are found in flaxseed, grains, nuts, fruits, and vegetables and isoflavones are found in soy products. A case-control study of the relationship between dietary intakes of lignans and risk of colorectal cancer showed that there was a significant reduction in colorectal cancer risk when comparing the highest intakes of lignan to the lowest = 0.71; 95% CI, 0.56 – 0.94, p value for trend = 0.01) (2).

It is thought that phytoestrogens may act via: *1*) hormonal effects mediated by ER (estrogen receptor) binding; *2*) non-hormonal actions by altering processes involved in carcinogenesis such as apoptosis and antioxidant activity; or *3*) interaction with enzymes involved in sex steroid biosynthesis and metabolism (38-40) . Isoflavones may alter

CYP(1A1,1A2,1B1)-mediated estradiol metabolism by reducing formation of carcinogenic hydroxylated metabolites while increasing less reactive 2-OH estrone and 16α-OH estrone metabolites (41). Also, phytoestrogens may inhibit CYP-dependent estrogen metabolism by acting as competitive substrates, or they may reduce circulating levels of estradiol by induction of CYP enzymes (2).

2.1.5 Meat consumption and colorectal cancer

Several studies showed that processed meat intake may be involved in the etiology of CRC with an estimated RR ~ 2 which is modest compared to RR of lung cancer due to cigarette smoking (relative risk (RR=20) (42-44).

Many carcinogens resulting from meat processing were suggested, the most likely being N-nitroso compounds (NOC) promoted by heme in the meat. This was supported by an associated increase in NOC-specific alkylating DNA adducts in colonic epithelial cells following by consumption of high meat diet (45;46). Other carcinogens such as Heterocyclic amines (HCA) and Polycyclic aromatic hydrocarbons (PCA) could be involved (47), however they are not specific for processed meat as they are found in significant amounts in chicken (48). Based on epidemiologic studies, risk for CRC was not significant for chicken meat consumption (49) .

2.1.5.1 Possible mechanisms relating red meat to CRC development:

Meat consumption is the major source of dietary arginine in humans, with high quantities of arginine found not only in red meat but also in pork, fish and chicken. Importantly, arginine is the key substrate for two competing metabolic pathways believed to be involved in carcinogenesis: the nitric oxide (NO) synthase pathway and polyamine synthesis. First, Arginine is catabolized by NO synthase 2 (NOS2) and other NO synthases to form nitric oxide. Inducible isoforms of NOS2 are abundant in human colorectal adenomas (50). All isoforms of NOS require five cofactors/prosthetic groups such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, BH4 and Ca2+-calmodulin. If NOS lacks its substrate, L-arginine or one of its cofactors, NOS may produce $^{\bullet}O_2^-$ instead of $^{\bullet}NO$ and this is referred to as the uncoupled state of NOS (51-54).

Second, Arginine is catabolized by the enzyme arginase to form ornithine, the substrate for putrescine synthesis. This is the first step in polyamine biosynthesis and is catalyzed by the enzyme ornithine decarboxylase (ODC) (55). Multiple abnormalities in the control of polyamine content results in increased polyamine levels that can promote tumorigenesis (56).

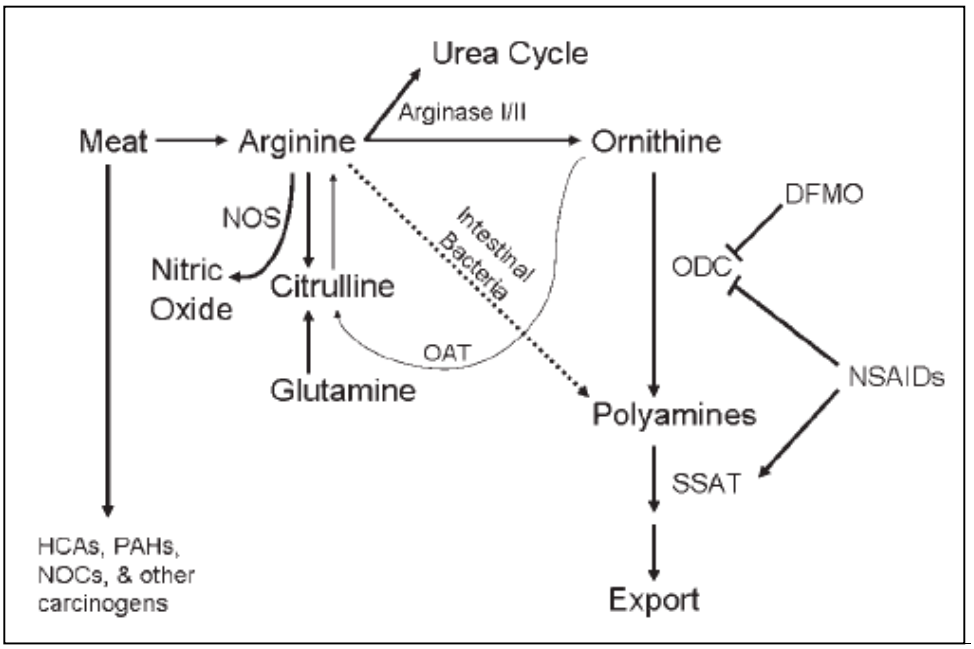

Figure (1**) Arginine synthesis, metabolism and catabolism.** Alternate pathways and inhibitors of these pathways are indicated. NOS, nitric oxide synthase; DFMO, difluoromethylornithine; ODC, ornithine decarboxylase; NSAIDs, non-steroidal anti-inflammatory drugs; SSAT, spermidine/spermine N^1-acetyltransferase; OAT, ornithine aminotransferase; HCAs, heterocyclic amines; PAHs, polycyclic aromatic hydrocarbons; NOCs, N-nitroso compounds (57).

Also, raw red meat contains high levels of oxymyoglobin and oxyhemoglobin on the surface of muscle, deoxymyoglobin and deoxyhemoglobin in the interior of muscle, and cytochromes of mitochondria in muscle and other tissues. The concentration of red color of the meat indicates the amount of mitochondria as well as the concentration of the major heme pigments. In cooked red meat, myoglobins, hemoglobins and cytochromes are converted into denatured protein-hemes, the hemichromes and hemochromes. After being eaten, heme proteins are hydrolyzed to amino acids and peptides and the heme group which is coordinated with strong ligands. The iron of heme coordinates to the sulfur, nitrogen or oxygen of amino acids and peptides and other biological components.

The coordinated heme groups are absorbed and transported by the blood to every organ and tissue (58). Free and coordinated heme preferentially catalyzes oxidative reactions such as lipid peroxidation. Heme catalyzed oxidations can damage lipids, proteins, DNA and other nucleic acids and various components of biological systems. Heme catalysis of oxidation is the strongest oxidizing system for developing tissue damage. These heme-catalyzed oxidations can lead to the initiation of biochemical and cellular damage and subsequently to disease processes (59) .

Evidence that heme from consumed red meat travels down the GI tract comes from studies of the occult blood test. The occult blood test detects hemoglobin and heme in stools from bleeding in the GI tract. That the occult blood test can give a false positive if a large amount of red meat is consumed shows that heme has been transferred through the GI tract (59).

2.1.6 Micronutrients and colorectal cancer:

2.1.6.1 Ca and vit D:

Calcium has been evaluated for its potential protective effect. Increased Ca intake is associated with decreased risk of colorectal cancer in epidemiological studies. Calcium has been associated with increasing cellular differentiation and apoptosis in both normal and tumor cells. For colorectal cancer, Ca may bind bile and fatty acids in the intestinal tract and thereby keep these compounds from damaging the intestinal mucosa (60).

Two intervention and six prospective studies provided information about the relationship between supplemental calcium intake and colorectal cancer reduction (61). One intervention study reported a significant reduction, however modest (RR=0.81), in recurrent colorectal polyps after supplementation with 1.2 g/day of Ca (62). The other intervention study recruited fewer subjects and thus was less powered. It reported reduced risk however not significant of colorectal cancer following calcium supplementation (63). Of the six prospectively designed observational studies, four reported some type of significant association (RR ranged from 0.76 - 0.6) between calcium supplements and the risk reduction of colorectal cancer, whereas two studies reported no association. Again, the studies that have reported protective associations for supplemental calcium were the cohorts with the largest number of subjects that contained both genders and a broad age range of subjects (64-69).

On the other hand, most epidemiological studies have reported that higher serum 25-hydroxyvitamin D levels are associated with lower incidence rates of various cancers (70-72). Almost all laboratory studies using tissue culture systems have reported inhibition of growth of malignant cells and many have identified re-differentiation in response to vitamin D metabolites especially 1, 25 $(OH)_2D_3$(73-78).

A profound inverse association of serum 25(OH)D with age standardized colorectal cancer mortality was found in the National Health and Nutrition Examination Survey III (NHANES) cohort. Individuals with serum 25(OH)D greater than 32 ng/ml had approximately one fourth the risk of dying of colon cancer as those with poor vitamin D status (<20 ng/ml) (RR= 0.28) (79).

The proposed molecular action of $1,25(OH)_2D_3$ on growth of colon cancer cells was demonstrated first by the finding that not only renal, but also cells from the heart, stomach, pancreas, colon, brain, skin, gonads and others have the nuclear receptor for $1,25(OH)_2D_3$, the so called vitamin D receptor (VDR), and such tissues are potential targets for $1,25(OH)_2D_3$ activity. Many of these VDR-positive tissues are known to be targets for development of sporadic malignancies (80). Bound to its receptor, $1,25(OH)_2D_3$ controls growth of normal and neoplastic cells and also controls cellular proliferation, differentiation and apoptosis (81-83). The relevance of VDR activation by $1,25(OH)_2D_3$ was investigated experimentally by using mice which were genetically altered to block $1,25(OH)_2D_3$/VDR signaling. The colon mucosa of VDR-null mice shows a pattern of increased DNA damage and cell division (84;85).

Dietary intake of vitamin D3 typically supplies only 10-20% of the daily requirement, whereas up to 80-90% comes from UV-B mediated synthesis of vitamin D3 in the epidermis (this depends on latitude and the season).

The preventive effects of higher vitD3 intake have led 16 vitamin D scientists in the United States and Canada to disseminate a call to action recommending universal daily intake of 2000 IU of vitamin D3 (72).

To maximize the efficiency of vitamin D in reducing the risk of colorectal cancer, it has been hypothesized that an optimal level of calcium has to be reached first. This was supported by a clinical trial of supplemental calcium (1400-1500 mg) with or without vitamin D3 (1100 IU) for lowering fracture risk in 1179 women. A statistically significant reduction in all cancer incidence (a secondary endpoint) occurred in the calcium plus vitamin D arm (OR=0.4) and a marginally significant reduction occurred in

the calcium alone arm. Another study found that 25(OH)D3 levels were associated with a reduced risk of adenoma recurrence only among subjects with high calcium intake (86).

The synergistic effect of calcium and vitamin D was not supported by a well-organized double-blinded clinical trial where patients were randomized to receive calcium carbonate (2000 mg/d) and / or vitamin D (800 IU/day) or placebo. They found that in normal colon mucosa, the apoptosis markers increased by a statistically significant 56% among individuals treated with vitamin D only. And these apoptosis markers increased by a statistically non-significant 33% in patients receiving calcium or calcium plus vitamin D. It was proposed that the combination of calcium and vitamin D was not as effective as vitamin D alone in promoting apoptosis. And possibly calcium did not add but also blunted the apoptotic effects of vitamin D (87). Apparently more research is needed to examine the molecular and the biological action of calcium and vitamin D on colon mucosa cells.

2.1.6.2 Folate and other one-carbon metabolism biomarkers

One-carbon metabolism reactions encompass a group of biological processes with two major functions: synthesis of purines and pyrimidines needed for DNA replication and repair and synthesis of S-adenosyl methionine, a methyl group donor for a number of methylation reactions including DNA methylation. 5-Methyl tetrahydrofolate provides the folate substrate for the remethylation conversion of homocysteine to methionine, the precursor to S-adenosylmethionine, and vitamin B12 serves as a cofactor for the reaction. The resulting tetrahydrofolate is converted to 5,10-methylene tetrahydrofolate in a

reaction requiring vitamin B2 Riboflavin, as flavin adenine dinucleotide, is the cofactor for methylenetetrahydrofolate reductase (MTHFR), the enzyme that influences homocysteine remethylation and DNA methylation. In a separate transsulfuration pathway, homocysteine is metabolized to cysteine via vitamin B6-dependent enzymes (88-90).

Low concentrations of these one-carbon related serum nutrients (i.e., folate, vitamun B6, vitamin B12, and riboflavin) may lead to elevated homocysteine, disrupted one-carbon metabolism, and insufficient methyl groups for DNA methylation, synthesis, or repair, thus potentially promoting carcinogenesis (89;91).

Experimental and human-based studies have linked folate to decreased colon cancer risk. Although some of these data suggest that folate and other one carbon markers should be protective, there is also evidence that folate's roles in cell signaling, may contribute to carcinogenesis. As folate is critical to cellular repair, folate-deficient or even depleted patients could well be at increased risk. On the contrary, a key mechanism of some chemotherapeutic agents is folate depletion. Epidemiologic evidence has tended to link folate to decreased risk. Nonetheless, clinical trials of folate supplementation have in general not confirmed these results. A recent clinical trial reported that supplementation with 1mg/day folic acid did not reduce but may have increased the recurrence of multiple and advanced colorectal adenomas (92;93). These results generated a hypothesis that folic acid might act in a bimodal way; deficiency may increase the risk of mutation, thus of tumerogenesis, but that supplementation may increase the survival and replication of mutated cells (94).

Similarly, studies investigating the role of vitamin B6 in colorectal cancer development are inconsistent, a recent meta-analysis concluded that dietary vitamin B6 intake and supplementation is inversely associated with colorectal cancer (95). However, a recent population-based prospective cohort design with a long follow-up period found that vitamin B6 is associated with increased risk of rectal cancer especially among women (91;96). The inconsistencies could be attributed to measurement error of food frequency questionnaire and non adjustment of unknown confounders (97).

2.1.6.3 B-carotene

Retinol (vitamin A_1) and its metabolites are essential in a wide range of physiological regulatory processes. At the molecular and cellular level, this regulation involves the control of cell proliferation and differentiation through retinoid-dependent effects on gene expression.

Epidemiologic studies, and laboratory experiments have suggested that carotenoids may control cell growth and may also play a role in carcinogenesis. Many studies observed strong associations between intakes of B-carotene rich foods and reduced risk of colorectal cancer. However, interest in B-carotene as a chemopreventive agent declined markedly as double blind, randomized clinical trials of B-carotene among average to high risk populations indicated that B-carotene probably increases the risk of some cancers and has no impact on the risk of several others (98). Results from recent intervention studies, the Beta-Carotene and Retinol Efficacy Trial [CARET] and the Alpha-Tocopherol, ß-Carotene Cancer Prevention Study [ATBC], indicated that exposure of

subjects taking supplemental ß-carotene to cigarette smoke increased lung cancer incidence (99).

2.2 Lifestyle Factors

2.2.1 Alcohol consumption and colorectal cancer

The risk of colorectal cancer was significantly associated with alcohol: individuals consuming the most alcohol had 60% greater risk of colorectal cancer compared with non- or light drinkers (relative risk 1.56, 95% CI 1.42-1.70)(6).

2.2.1.1 Mechanisms relating Alcohol to CRC development:

Although alcohol itself is not carcinogenic, alcohol's first metabolite, acetaldehyde, is emerging as an important mal-factor, being able to form stable DNA adducts, trigger mutations in tumor suppressors and oncogenes, and to interfere with DNA repair (100).

The pathological effects of alcohol consumption on the colorectal tract have been extensively investigated but still remain largely unclear. Evidence exists for the involvement of acetaldehyde (101), but also other mechanisms have been linked to CRC, e.g. the induction of reactive oxygen species through the induction of cytochrome P-450 2E (102-104). Other molecular changes seen in CRC involve alterations in DNA methylation, e.g. induction of expression of oncogenes and silencing of tumour suppressor genes (105). Many of the above-described processes have been observed in heavy or chronic alcohol consumers. The amount of damage related to alcohol tends to

follow a dose–response relationship and, as such, (very) high levels of daily alcohol intake may be needed for genetic mutations to occur.

2.2.2 Physical activity:

There is compelling evidence linking physical inactivity and obesity with an increased risk of developing colon cancer. A recent meta-analysis was conducted. The meta-analysis included only longitudinal observations where current weight, height and leisure time exercise were assessed before the diagnosis of cancer and did not rely on retrospective recall of past habits. They show that higher levels of leisure time-physical activity are associated with modest reductions (14-20%) in risk of colon cancer in men and women with a dose response relationship, but no apparent reductions in risk of rectal cancer in either men or women (106). This is in general agreement with other studies; however, the magnitude of risk reduction may differ (107-110). This meta-analysis also showed that the protective effect of physical activity decreased after adjustment for BMI especially in men (RR before BMI adjustment= 0.58, RR after BMI adjustment=0.80) (106). If obesity is considered as a marker of prolonged excess of energy intake, this suggests that physical activity and BMI are not independent from each other.

Several hypotheses provide an interpretation for the obesity- cancer relationship. The hyperinsulinemia hypothesis states that chronic elevated insulin promotes the proliferation of colonic cells including cancerous clones. The effect can be direct or indirect by increasing circulating levels of free insulin-like growth factors. The same mechanism could explain the protective effect of physical activity which reduces

circulating levels of insulin. This was supported experimentally in animal studies (111).

To explore the effect of calories on carcinogenesis in greater detail, several animal studies confirmed the inhibitory effect of caloric restriction on tumorigenesis. One study examined the effect of 40% restriction of calories in rats, where absolute saturated fat intake was increased. Interestingly, this study demonstrates that the tumor-reducing effect of calorie restriction dominates even if the fat intake increases during calorie restriction. Thus total energy is an important determinant in colon carcinogenesis. Whether caloric restriction is achieved by dietary means or through increased consumption by exercise, whichever is more efficient, needs to be further investigated in human studies (112).

2.2.3 Smoking and Colorectal cancer;

Several studies have linked cigarette smoking and alcohol intake to many cancers. The association of cigarette smoking and colon cancer is much smaller than that between smoking and lung cancer. Nonetheless, the impact of tobacco smoking on colon cancer risk is evident. A meta-analysis of prospective cohort studies show that current male smokers carry 38% increased risk of colorectal cancer than non smokers..

2.2.4 Obesity and colorectal cancer

The pooled estimate indicated that individuals with a BMI ≥30 kg/m2 had a 40% greater risk of colorectal cancer compared with individuals with a BMI ≤25 kg/m2(6).

2.2.4.1 Positive energy balance (PEB) and obesity in relation to CRC:

Excess weight has been associated with increased mortality from all cancers combined and for cancers of several specific sites. Overweight and obesity are clinically defined indicators of a disease process characterized by the accumulation of body fat due to an excess of energy intake (nutritional intake) relative to energy expenditure (physical activity). When energy intake exceeds energy expenditure over a prolonged period of time, the result is a positive energy balance (PEB), which leads to the development of obesity. This physical state is ideal for intervention and can be modulated by changes in energy intake, expenditure, or both (113).

The outcome of PEB, excess adipose tissue, has two main categories: subcutaneous and visceral. Subcutaneous adipose is defined as fat tissue between the skin and muscle, whereas visceral adipose tissue is found within the main cavities of the body, primarily in the abdominal cavity. Abdominal visceral adipocytes are more metabolically active and linked to a series of reactions leading to carcinogenesis (114).

Several measures are used to define obesity. The most utilized parameter is body mass index (BMI) (weight in kilograms divided by height in meters squared), a measure of overall overweight status. The World Health Organization (WHO) defined an overweight status as a BMI of 25.0 or higher. Only two techniques can distinguish between subcutaneous and visceral fat abdominal adipose tissue. They are computed tomography (CT) and magnetic resonance imaging (MRI). These two techniques are too costly and complex to be used in large-scale epidemiologic studies. Therefore, the waist-

to hip ratio (WHR), the circumference of the waist and hip, is used as a proxy measure of central adiposity (115).

Studies in animals showed that restriction of calories by 10 to 40% has been shown to decrease cell proliferation, increasing apoptosis through anti angiogenic processes(115).

2.2.4.2 Mechanisms Relating Positive Energy Balance to Cancer Risk

Many studies have tried to explain the mechanisms which link obesity with colorectal cancer, among them are: Insulin resistance, chronic inflammation, and the adiponectin theory.

Insulin resistance and CRC development. The term insulin resistance refers to a state of cellular unresponsiveness to the effects of insulin with higher levels of insulin required to normalize plasma glucose. Insulin resistance is believed to underlie a cluster of metabolic abnormalities including elevated levels of blood triglycerides and glucose, low levels of high-density lipoprotein cholesterol and high blood pressure (116).

At least three mechanisms exist through which insulin resistance potentially causes colorectal cancer (116). The elevated concentrations of plasma insulin, triglycerides, NEFA and glucose associated with insulin resistance lead to increased insulin exposure of non-classical insulin target tissues that express insulin receptors, such as the colon. This can potentially have a number of consequences. First, insulin is known to have growth as well as metabolic effects. Specifically, insulin stimulates proliferation

and reduces apoptosis in colorectal cancer cell lines (117;118), and it promotes colorectal tumor growth in animal models (119-121). Because the colon is not a classical insulin-target tissue, the colonocyte may lack a specific mechanism through which the mitogenic actions of insulin are regulated, as is the case in classical insulin target tissues such as skeletal muscle, adipose tissue and liver. Thus, elevated insulin signaling in the colonocyte may engender an enhanced proliferative state with tumorigenic consequences (122).

Second, in conjunction with the metabolic effects of insulin, the increased concentrations of available energy substrates such as glucose, triglycerides and NEFA may provide increased energy for transformed colonocytes as well as induce changes in cell signaling pathways. Elevated intracellular levels of triglycerides and their metabolites such as diacylglycerol may activate the protein kinase-C and MAPK pathways with potentially mitogenic and carcinogenic effects (123). Triglycerides and other fat metabolites are known to affect the activity of peroxisome proliferator activated receptors (PPARs), a class of transcription factors that play key roles in lipid, glucose and energy homeostasis and in adipocyte differentiation regulation. PPARs have antiproliferative, proapoptotic and anti-inflammatory effects (124). Peroxisome proliferator-activated receptor gamma (PPAR-g) is expressed in colonic tissue and inhibits the growth and increases the differentiation of colonic tumors (125). Increased energy availability may also contribute to colon carcinogenesis by stimulating reactive oxygen species synthesis. An intracellular lipolytic environment rich in oxidizable substrates may result in the generation of lipid oxidation products, depleted levels of antioxidants and an overall environment of oxidative stress (126).

Hyperglycemia may also increase oxidative stress (127). In support of this, DNA damage is known to be higher in diabetic individuals compared with healthy subjects (128).

Third, insulin resistance causes alterations in the IGF system with concomitant effects on cellular growth pathways. Insulin and IGF are representative of energy availability and stimulate anabolic pathways, leading to cell growth and differentiation. In the hyperinsulinemic state, IGF-binding protein (IGFBP) levels decrease, whereas free IGF-1 levels rise (129). The colon expresses IGF receptors, and following activation by IGF binding, colonocyte apoptosis is inhibited and cell cycle progression ensues. Elevated levels of IGF may therefore provide a selective growth stimulus, causing clonal expansion of epithelial cells with abnormal growth regulation. High circulating levels of IGF-1 have been positively associated with colorectal cancer risk, whereas high IGFBP-3 levels are associated with reduced risk (130;131). Furthermore, sufferers of acromegaly, a condition characterized by overproduction of IGF and growth hormone (GH), have increased risk of developing colorectal cancer (132).

Inflammation. Overweight and obese individuals experience low-grade systemic inflammation. IL-6 is secreted by visceral adipose tissue, in vivo, particularly in obese individuals. Long-term secretion of IL-6 dampens the production of Tumor Necrosis Factor-α (TNF-α) and stimulates C-Reactive Protein (CRP) (133).

A recent cohort study conducted in older adults aged 70 – 79 years found that elevated baseline levels of IL-6, CRP, and TNF-α were associated with an increased risk of cancer events. Colorectal cancer risk was associated with IL-6 and CRP, which is consistent with findings from another nested case-control study (134;135) .

Adiponectin. Adipose tissue, which is the largest endocrine organ in the body, plays an important role in regulating energy metabolism and inflammation, and has also been associated with several cancers. Adiponectin, a 30-kDa complement C1-related protein, is the most abundant gene product secreted by fat cells (136) and is a key regulator of insulin sensitivity (137;138) and inflammation (139;140). Adiponectin modulates several physiologic processes, such as metabolism of glucose and fatty acids (141), and decreased plasma adiponectin concentrations are associated with insulin resistance, type 2 diabetes (142), and atherosclerosis (143). In addition, it has recently been shown that adiponectin may play a role in the development and progression of various types of malignancies(144).

A large, prospective, nested case-control study found that plasma adiponectin concentrations were inversely associated with risk of colorectal cancer in men (145) . Men with the highest concentrations had ~60% reduced risk for colorectal cancer compared with those with the lowest concentrations, even after adjustment for body size, waist circumference, and physical activity (145). In addition, it was found that colorectal tumors express adiponectin receptors, and that this expression is significantly higher than in non-tumorous colorectal tissue from colorectal cancer patients. The elevated expression of AdipoR1 and AdipoR2 further indicate a potential role of adiponectin in the pathogenesis of colorectal cancer (144).

2.2.5 Cigarette smoking and colorectal cancer

In a meta-analysis of prospective cohort studies, the risk of CRC among male smokers (RR, 1.38; 95% CI, 1.22-1.56) was more significant than among female smokers (RR, 1.06; 95% CI, 0.95-1.19). Former smokers still carried a higher CRC risk than subjects who never smoked. The increased risk of CRC was related to cigarettes per day, longer years of smoking, or larger pack-years (146).

2.2.6 Antiinflammatory drugs and CRC

Much data indicate that long-term users of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) reduces colon cancer risk by 40–50%, The ability of NSAIDs to inhibit cyclo-oxygenases (COX-1 and -2) underlies their mechanism(s) of chemoprevention (147). COX-2 converts arachidonic acid to prostaglandins, which in turn induce inflammatory reactions in damaged tissues. Aspirin irreversibly inactivates both COX-1 and COX-2. Inactivation prevents platelet synthesis of prostaglandins, endoperoxides and thromboxane A2 (148).

2.3 Previous Medical Interventions

2.3.1 Pelvic Irradiation

Previous exposure to pelvic radiation may be associated with a higher risk of CRC after a 5- to 10-year lag period (149). A history of radiation therapy for prostate cancer correlated with an increased risk of rectal cancer in a large retrospective study (150).

2.3.2 Ureterocolic Anastomosis

A few studies have reported an apparently increased risk of colorectal neoplasia near ureterocolic anastomoses after major surgery of the urinary or intestinal tract (151;152). The presumed mechanism is exposure of colonic mucosa to carcinogenic substances from the urinary system.

2.4 Concurrent Medical Conditions

2.4.1 Inflammatory Bowel Disease (IBD)

Inflammatory bowel diseases include at least two forms of intestinal inflammation: Ulcerative colitis and Crohn's disease, the causes of which are unknown. Ulcerative colitis and Crohn's disease are distinguished by their typical clinical, pathologic, radiologic, endoscopic, and laboratory features (153).

Patients with chronic IBD involving the colon are at significantly increased risk for CRC (154;155). In ulcerative colitis (UC), the risk of CRC depends on the duration and extent of disease. The risk is highest in those with extensive colitis or " pancolitis, " with a standardized incidence ratio of 2.4 according to a population-based study in the USA (156).

More recent studies have reported that the risk of CRC in long-standing Crohn's disease (CD) involving the colon is probably comparable to that of UC (157-160). CRC

in CD develops over a similar time frame as in UC (161;162). The mean age of onset for IBD-associated CRC is lower than that for sporadic CRC (45 versus 60 years).

Although inflammatory mechanisms have been associated with the development of colon cancer that arises either sporadically (135;163;164), or with IBD, the link to inflammation is stronger in IBD-associated carcinogenesis (165).

By comparing the different mechanisms leading to colon cancer, the role of inflammation in carcinogenesis has been investigated. In both sporadic and IBD-associated colon cancer, carcinogenesis is associated with genetic instability arising from both chromosomal instability and microsatellite instability. However, the clinical features of these two types of colon cancer differ(165). In sporadic cancer, a progression from adenoma to carcinoma is apparent, with the dysplastic precursor generally localized to a discrete focus. In contrast, IBD-associated carcinogenesis results when the epithelium in areas of inflammation progresses to dysplasia and then to carcinoma, with dysplastic lesions generally appearing as multifocal and diffuse. In addition, the timing and frequencies of molecular alterations in sporadic and IBD associated colon cancer differ (165). Molecular alterations that contribute to the development of sporadic colon cancer include the loss of chromosomal material (loss of heterozygosity), microsatellite instability, and aberrant DNA methylation. In IBD-associated colon cancer, these mechanisms are also implicated but they generally occur simultaneously and early in the progression to carcinogenesis. The early occurrence of these three common factors suggests inflammation plays a causative role (166).

2.4.1.1 Mechanisms relating Chronic Inflammation to CRC:

The gastrointestinal tract is continuously exposed to various environmental antigens, including not only the beneficial microbes residing there, but also a wide variety of toxins and pathogens (167). Therefore, it is important that an efficient immune system is present along the entire length of the gut.

Tissue inflammation such as gastritis, esophagitis, and hepatitis, which are caused not only by chronic infection but also by physical and chemical agents such as heat, acid, UV, tobacco smoke, and foreign bodies (e.g., asbestos), are also recognized risk factors for human cancer at various sites. In addition, autoimmune and inflammatory reactions of uncertain etiology (e.g., pernicious anemia, ulcerative colitis, pancreatitis, etc.) are associated with increased risk of cancer.

The mechanisms of carcinogenesis associated with infection and inflammation have not been fully elucidated. Three main mechanisms have been proposed to account for infection-associated carcinogenesis: First, direct action of the infectious agent on host cells or tissues which leads to alterations of host DNA (insertion, deletion, translocation, and amplification). Tumor-suppressor gene products such as pRB and p53 are also inactivated by interaction with products of integrated viral DNA (e.g., the X-protein of hepatitis B virus, the E6 and E7 proteins of human papillomavirus) .Second, viral infection (e.g., with human immunodeficiency virus) may cause immunosuppression, which can enhance some types of malignancy (e.g., Kaposi's sarcoma). And third, it was found that prolonged activation of inflammatory cells generates ROS and RNS that can damage host DNA and tissues and contribute to carcinogenesis (168).

Production of ROS and RNS in inflammation induced cancer. Infection and inflammation activate a variety of inflammatory cells, which induce and activate various oxidant-generating enzymes . These include NADPH oxidase and xanthine oxidase, which produce superoxide anion ($O^{\bullet -2}$), inducible nitric oxide synthase (iNOS), which produces nitric oxide ($NO^{\bullet}$) from L-arginine, and myeloperoxidase (MPO), which generates hypochlorous acid (HOCl) using hydrogen peroxide (H_2O_2) and chloride ion (Cl^-) as substrates. Peroxidases such as MPO can also catalytically generate nitrogen dioxide ($NO^{\bullet}{}^2$) using H_2O_2 and nitrite as substrates (169;170).

Under normal conditions, free radicals have a role in body defense mechanism against bacteria and viruses (171).

Free radicals may react with phospholipids of membranes generating hydroperoxides, lipoperoxides and toxic aldehydes such as malondialdehyde (MDA), which in turn may alter membrane permeability and microcirculation. ROI, RNI and their derivative products may also activate nuclear factors such as, for example, NF-KB, leading to the production of other proinflammatory cytokines, which in turn enhance inflammation and, therefore, the generation of more reactive species (172). The imbalance in redox status and the enhanced production of intermediate reactive species progressively consumes the antioxidant defenses, leading the cells to develop oxidative stress. Cells could respond to these insults by enhancing their antioxidant potential or by activating the system of caspases that induce programmed cell death (apoptosis). It is also possible that oxidative stress induces cell necrosis through the release of cytochrome c and the depletion of ATP at the mitochondrial level (173;174). Peroxynitrite participates with the apoptotic

program through the nitrosylation of proteins, which in turn alters the function of several signaling molecules including NF-KB, p53 and caspases (175-177). When the system fails in one or more than one step, the possibility of developing a mutated cell increases as a consequence of the high perturbation in the redox status that progresses, through ROI and RNI, into the nucleus (178).

On the other hand, nitric oxide (NO) is generated specifically during inflammation via iNOS in inflammatory and epithelial cells. Excess NO production plays a crucial role in cancer (168). NO reacts with superoxide ($O^{\bullet -2}$) to form peroxynitrite (ONOO-), a highly reactive species causing nitrative and oxidative DNA damage. ONOO- can mediate the formation of 8-Oxo-2'-deoxyguanosine (8- oxodG) (179) and 8-nitroguanine, a marker of nitrative DNA damage (180;181). 8-Nitroguanine is considered to be not only a marker of inflammation, but also a potential mutagenic DNA lesion product, leading to carcinogenesis (182).

2.4.2 Diabetes Mellitus type 2

DM is a group of metabolic disorders characterized by hyperglycemia. The two most frequent subtypes of diabetes mellitus differ for both metabolic and hormonal characteristics: in type 1 diabetic patients (5-10% of all diabetics) hyperglycemia is associated with an absolute deficiency of endogenous insulin secretion and the absolute requirement for exogenous insulin administration.

In type 2 diabetes hyperglycemia and hyperinsulinemia coexist for a long time because of the insulin resistance of peripheral tissues. Only when the beta-cell function

fails completely, will the patient require insulin treatment because of endogenous insulin deficiency (183).

Type 2 diabetes has been associated with an increased risk of colorectal adenomas and carcinomas in most, but not all, studies. The risk is increased in both women and men for both colon and rectal cancer (184).

Hyperinsulinemia has been suggested as a link between CRC and diabetes as insulin is a growth factor not only with metabolic, but also mitogenic effects, and its action in malignant cells is favored by mechanisms acting both at the receptor and post-receptor level cells (185;186). Chronic insulin therapy has been associated with an increased colorectal tumor risk among type 2 diabetic patients (187). Specifically, a three-fold risk increase for patients with insulin-dependent type 2 DM in comparison to the general population has been observed (188).

In most type 2 diabetic patients hyperglycemia is associated with endogenous hyperinsulinemia, a compensatory state caused by insulin resistance. This condition often persists for many years. Therefore, in these patients, excess unused substrates (i.e. glucose) are present concomitantly with hyperinsulinemia. This abnormal situation is accompanied by a series of other abnormalities involving other hormones, like glucagon, incretins, leptin, etc.

As DM persists for many years, most type 2 diabetic patients progress from progressively decreased insulin secretion to failure of B-cells to secrete insulin.
At this stage, patients with type 2 diabetes may become similar to type 1 diabetic individuals (189).

If hyperinsulinemia has a role in promoting cancer initiation and/or progression, these aspects should be considered when determining the individual risk of a diabetic patient to develop cancer. Most studies on the diabetes-cancer association overlooked these different biological conditions. Therefore, it is inappropriate to consider diabetic patients as a homogenous cohort. In conducting a new study, a number of confounders should be adjusted for first; endogenous insulin status, obesity, quality of metabolic control and drugs used for treatment (190).

2.4.3 Human Immunodeficiency Virus Infection

Some studies have shown an increase in the incidence of colorectal neoplasia in patients infected with the human immunodeficiency virus (191). This is thought to be a result of increased susceptibility to carcinogenesis due to chronic immunosuppression.

2.4.4 Allergy:

In a prospective study involving 21,292 Iowa women followed for 8 years allergy was defined from four self-reported questions about physician-diagnosed as asthma (a), hay fever (b), eczema or allergy of the skin (c), and other allergic conditions (d). It was found that history of any allergy was inversely associated with incident colorectal cancer: after multivariate adjustment, the hazard ratio (HR) was 0.74 [95% confidence interval (95% CI), 0.59-0.94] compared with women with no allergy (192).

The observed inverse associations, if causal, may reflect enhanced immunosurveillance in allergic participants (i.e., the enhanced ability of the immune system to detect and eliminate cancer cells before they become clinically manifest). This

hypothesis is consistent with findings in other epidemiologic studies that showed decreased risk estimates for various cancers, such as pancreatic cancer, liver cancer, glioma, and breast cancer, associated with a history of allergy (193-198). The hypothesis that allergies lead to enhanced immunosurveillance is supported by laboratory studies that show that allergy is accompanied by immunoglobulin E production, a significant decrease in tumor occurrence and growth, and an increase in survival time in sensitized mice (199-201).

2.4.5 Acromegaly

A prospective controlled study found that adenomatous polyps occurred in 22% of male acromegalic patients compared with 8% of control subjects (202). Patients with acromegaly were more likely to have multiple and proximal adenomas. Reduced expression of the PPAR gene has been implicated in such patients (203).

3 Molecular basis of Colorectal cancer

3.1 Introduction:

Three major categories of genes have been implicated in carcinoma development: (1) oncogenes, (2) tumor suppressor genes, and (3) mismatch repair genes

Type	Name	Chromosome
Oncogen	K-*ras*	12
Tumor suppressor gene	*APC*	5
	DCC	18
	p53	17
	MCC	5
	TGF-β-RII	3
Mismatch repair gene	*hMLH1*	3
	hMSH2	2
	hPMS1	2
	hPMS2	7
	hMSH6	2
	hMSH3	5
Others (currently of theoretical importance only)	Fat acetylation *p450* genes, etc.	Many

Table (1) **Genes Known to Be Involved in Development of Colorectal Carcinoma** (204).

When a proto-oncogene (a normal human growth related gene) becomes abnormally activated, it drives the cell through the cell cycle facilitating clonal proliferation and is known as an *oncogene*. Oncogenes act in a dominant fashion because alteration of only one allele is necessary to produce a cellular effect.

Other genes called *tumor suppressor genes* can halt the cell cycle even when oncogenes are altered. Tumor suppressor genes act in a recessive manner and promote carcinoma only when they are inactivated by allelic loss or mutations in both alleles. If cells cannot repair DNA damage, tumor suppressor genes such as p53 drive the cell into a suicide mode called apoptosis.

The latest genes found to be related to carcinogenesis are called *mismatch repair genes*, which are needed for cells to repair DNA replication errors and spontaneous base pair loss (204).

3.2 Genes involved and Genetic pathways in colorectal cancer:

There are two major genetic mechanisms responsible for colorectal carcinogenesis. Most likely there are other mechanisms yet undiscovered. First, the chromosomal instability (CIN) pathway, the accumulation of mutations in tumor-suppressor genes and oncogenes, as well as other epigenetic changes such as hyper- or hypomethylation of DNA, that drive the cells to become malignant. This pathway is referred to as the suppressor pathway or chromosomal instability pathway. Second, the mutator pathway in which inactivation of the mismatch repair genes leads to the accumulation of mutations (insertions or deletions) in microsatellites. These are DNA repetitive sequences located throughout the genome. These microsatellite mutations may lead to genomic instability, which, in turn, may accelerate further accumulation of mutations in other cancer genes during tumorigenesis(205).

The common theme of these pathways is genomic instability. The result of genomic instability is the accumulation of mutations that provide a survival advantage to specific clones of cells, which eventually could become carcinogenic. These mutations occur in genes that control cell growth and cell death (206).

Tumors developing along the mutator pathway are characterized by microsatellite instability and, in general, are diploid, whereas tumors originating via CIN are usually aneuploid.

3.2.1 Genes Involved in CIN

Mutations in the APC gene are common in both pre-malignant and malignant colorectal neoplasms. Germ line mutations in the APC gene are characteristic of FAP (Familial Adenomatous Polyposis). The APC gene is located in chromosome 5q21. It consists of 16 exons encoding a 2,861-amino-acid protein (207). Somatic mutations in the APC gene occur in sporadic colorectal cancers, whereas in FAP, a germ line mutation is inherited and a somatic mutation acquired. As occurs in FAP, in sporadic colorectal cancer, the second mutation depends on the site of the first mutation (208). Depending on the location of the first hit, the second hit may be an allelic loss or another truncating mutation (209).

K-ras is a proto-oncogene involved in signal transduction (210). Oncogenic mutations in k-ras allow k-ras to remain activated and to interact with downstream signaling molecules to stimulate cell proliferation. Even though k-ras mutations have been identified in aberrant crypt foci, they are rarely seen in adenomas smaller than 1 cm

(211). They are common in larger adenomas and in colorectal carcinomas, which suggests that k-ras mutations occur later in the pathogenesis of colorectal cancer (212).

The TP53 gene is located in chromosome 17p. The p53 protein is a transcription factor with tumor-suppressor properties (210). Activated p53 will result in transcription of genes that regulate cell-cycle progression, apoptosis, and inhibition of angiogenesis by induction of thrombospodin-1 (TSP1) (213).

The deletion in colon cancer (DCC) gene is located in chromosome 18q. Mutations in DCC are more frequent as adenomas progress to carcinomas. (212).

3.3 Genetic Pathways:

The concept of multi-step 'adenoma–carcinoma' sequence involving the inactivation of APC (the 'gatekeeper') and the subsequent stepwise mutation of several other genes such as K-ras and p53 have been proposed in the past decades (214).
One critical observation is the finding that nearly 70% of sporadic (average-risk) colorectal adenomatous polyps harbor somatic *APC* mutations (215).

3.3.1 Loss of Heterozygosity :

APC gene inactivation leads to a pathway termed loss of heterozygosity (LOH). Approximately 70% to 80% of colorectal carcinoma develops through the LOH pathway following inactivation of the APC gene. The genes involved in the LOH pathway include K-ras, DCC, and p53 in addition to APC. Germline APC mutations initiate the neoplastic process in patients with familial adenomatous polyposis (FAP) and endow all colonic crypt stem cells with a high risk for clonal proliferation.

The cascade of events begins with a loss or mutation of the APC gene on chromosome 5q, resulting in a change from normal epithelium to hyperproliferative epithelium. One of these hyperproliferating cells gives rise to a small adenoma in which the genome is hypomethylated. The next event involves activation of the K-ras oncogene on the chromosome 12p mutation to form the intermediate adenoma. Unlike oncogenes, tumor suppressor genes are expressed in a recessive manner. Therefore, both allelic copies must be lost or inactivated by point mutations for phenotypic expression to occur. Usually the DCC gene on chromosome 18q is next to be deactivated or lost, and results in the development of a late adenoma. The final genetic alteration found consistently in colorectal carcinoma is loss and/or mutation of the p53 tumor suppressor gene on chromosome 17p. While there is no obligatory sequence of mutations in the pathway from normal mucosa through adenoma to carcinoma, there is clearly an association of certain types of mutations in specific oncogenes or tumor suppressor genes with early and late states of transformation.

This multistep pathway can be observed both in sporadic and inherited colorectal carcinoma. Many other genes, such as MCC, TGF-b, and Myc, have been implicated in the genesis of colorectal carcinoma (212).

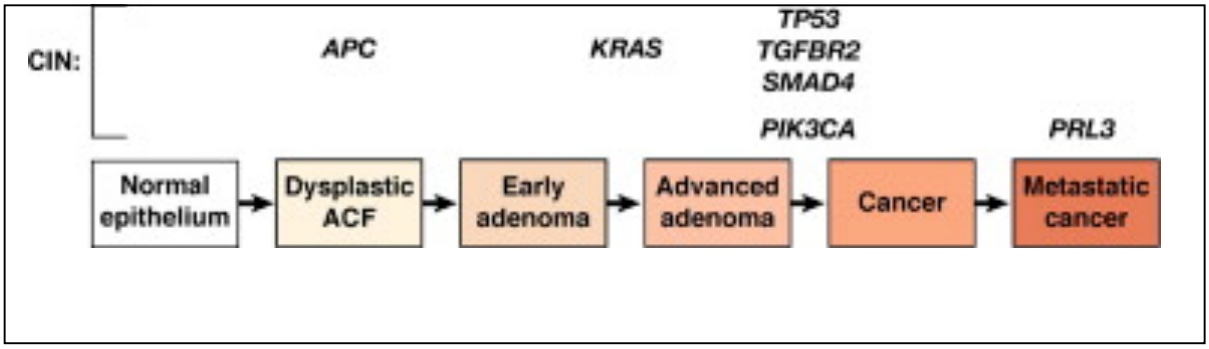

Figure (2) **Progression of colorectal tumors with CIN. The hallmark of the CIN pathway is aneuploidy.** Initiation of neoplasia in this pathway occurs by a somatic mutation in one allele and loss of heterozygosity of the second normal allele of the APC gene. Progression is then driven by successive waves of cellular clonal expansion that acquire enhanced growth characteristics and include mutational activation of the proto-oncogene KRAS and mutation of TP53 with subsequent loss of heterozygosity of the normal remaining TP53 allele to allow carcinoma formation (210).

3.3.2 Mutator Pathway, MSI (Microsatelite Instability):

Colorectal tumors that develop through this pathway are initiated by inherited (HNPCC) or somatic mutations within one of the DNA mismatch repair genes namely, hMSH2, hMSH3, hMSH6, hMLH1, hPMS1, and hPMS2. Epigenetic silencing of the hMLH1 promoter is another mechanism of inactivating hMLH1(4). Colorectal tumors generated via the mutator pathway are characterized by microsatellite instability. MSI has been defined as microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L), and microsatellite stable (MSS), depending on the number of unstable loci (216).

Microsatellite instability is involved in the genesis of about 15% of sporadic CRCs and most of hereditary non-polyposis CRCs (HNPCC) (217;218). The multiple errors in repetitive DNA sequences (microsatellites) result from a failure of the DNA mismatch repair (MMR) system to edit errors made during DNA replication. The DNA

MMR system is inactivated either by hypermethylation of the promoter, which silences gene transcription of hMLH1 (epigenetic phenomenon; sporadic CRC), or because of germ-like mutations in MMR genes MLH1, MSH2, MSH6 and others (genetic phenomenon, HNPCC) (4).

Mismatch repair gene defects initiate an entirely different sequence of events known as the replication error (RER) pathway. These pathways lead to carcinomas that are biologically quite different. This second pathway to colorectal carcinoma is found in approximately 20% of carcinomas.

Patients with HNPCC inherit a single defective allele of a mismatch repair gene and require an additional somatic mutation to inactivate the second allele. Spontaneous carcinomas develop after two somatic events inactivate the relevant gene. In either case, inactivation leads to a marked increase in replication errors. As errors accumulate in microsatellites, malfunction of genes that contain or are near affected microsatellites may occur. RER-positive phenotype was found in 77% of colorectal carcinomas from HNPCC patients compared with only 13% of patients with sporadic carcinoma (219).

Each pathway appears to prevail in a different colorectal area, and to be associated with a different histotype and prognosis: the CIN pathway in tumors of the left/distal colon, which are usually aneuploid , highly differentiated, rarely mucinous, with no lymphocyte infiltration; the MSI pathway in tumors of the right/proximal colon, which are diploid, poorly differentiated, often mucinous, with Crohn-like lymphocyte infiltrate (220).

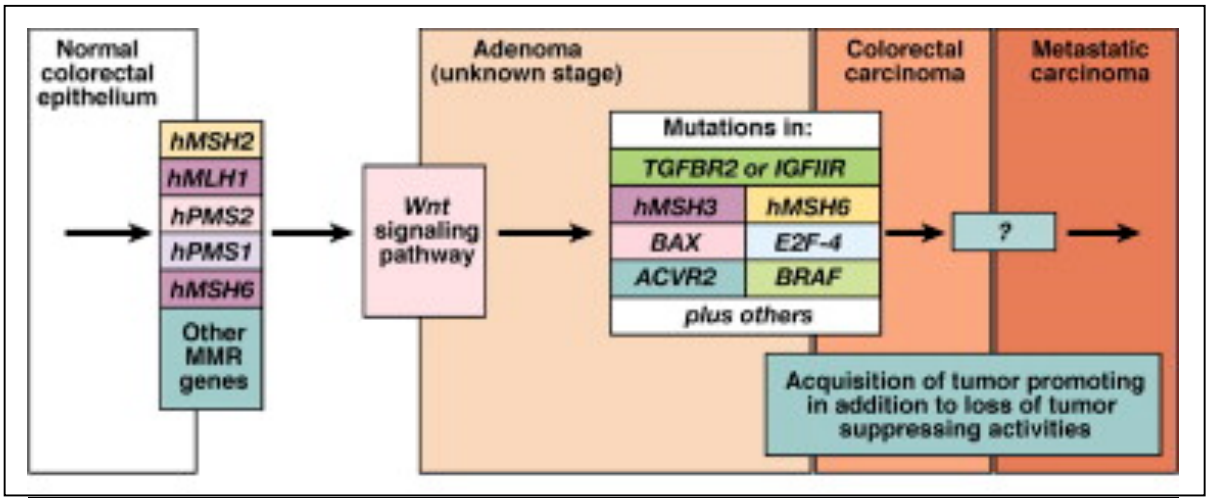

Figure (3) **Progression of colorectal tumors with MSI.** MSI tumors, whether sporadic or from patients with Lynch syndrome, lose MMR function early in the polyp → cancer progression sequence. Sporadic tumors almost uniformly lose MMR function due to hypermethylation of the promoter of *hMLH1*, whereas patients with Lynch syndrome have a germline mutation in one of the MMR genes (210).

3.3.3 Epigenetic alterations and Epigenetic instability:

Epigenetics (from the Greek "upon" genetics) is a modification of the genome, as opposed to being part of the genome.

The diversity in cancer cell populations is believed to be due to: (a) accumulation of genetic changes (e.g., mutations) that lead to differential gene expression, thereby allowing for uncontrollable cell growth, and (b) epigenetic (stable) heritable changes in gene expression mechanisms (not attributable to nucleotide sequence variation) that describe the interactions of genes with the environment. Most CRCs have two main epigenetic abnormalities: DNA methylation and core histone modifications, which

coexist with more classical genetic changes such as p53, k-ras and β-catenin mutations. These epigenetic effects have an important role in development, but can also arise as individuals age (221).

DNA is wrapped around a core of eight histones to form nucleosomes (the smallest structure unit of the chromatin) that function as DNA packaging units and as transcriptional regulators. The amino terminal tail of histones protrudes out from the nucleosome and are subject to posttranslational chemical modifications such as phosphorylation, ADP-ribosylation, glycosylation, biotinylation, acetylation by histone acetyltransferases (HATs), and methylation by histone lysine methyltransferases (HMTs). Unlike histone lysine acetylation, histone lysine methylation can result in either activation or repression, depending on the residue on which it resides; in this way, specific modifications of histone tail residues can be used as markers of transcriptionally active or inactive chromatin (222). Histone modifications affect the access of regulatory factors and complexes to chromatin, thereby influencing gene expression (223).

Aberrant methylation of DNA (global hypomethylation of promoter regions of genes accompanied by region-specific hypermethylations) is frequently found in tumor cells. Global hypomethylation leading to overexpression of oncogenes can result in chromosomal instability (CIN) present in >50% of colorectal cancers (CRCs) (224). Methylation occurs via the covalent addition of a methyl group to the 5- position of the cytosine ring within the context of a cytosine nucleotide followed by a guanine nucleotide (CpG dinucleotide). The term CpG island methylation phenotype (CIMP+) was introduced several years ago to describe tumors that display frequent and concurrent

hypermethylation of multiple CpG islands in new and known genes, including the mismatch repair gene human Mut L homolog 1 (hMLH) (225) .

Hypermethylation also occurs in the aging process and these acquired changes may dispose elderly individuals to neoplasia. For example, an increase in methylation of the estrogen receptor (ER) gene in normal colonic tissue, as well as in highly methylated colon tumors, as a function of age was reported. Age-related methylation and subsequent inactivation of tumor suppressor genes have been suggested as a predisposing factor for the increased risk of cancer with age (226).

3.4 Clinical syndromes:

The APC and mismatch repair genes were found to initiate FAP and HNPCC, the two main autosomal dominantly inherited CRC's, respectively (227). More recently, different initiating genes have been found for other familiar CRC syndromes (228).

3.4.1 Inherited syndromes:

3.4.1.1 Lynch syndrome or HNPCC

The most common form of hereditary CRC is Lynch Syndrome (LS), also known as hereditary non-polyposis colorectal cancer (HNPCC), which accounts for 2–5% of the total CRC burden (229).

Heterozygous germ-line mutations in the DNA MMR genes MSH2 and MLH1 are responsible for most HNPCC families, while MSH6 and PMS2 are less frequently

involved (230). Tumors arise as a result of somatic inactivation of the same MMR gene that is mutated in the germline.

The name LS is used for families with a verified MMR gene mutation. Families with clinical features of LS but no molecular genetic verification are known as HNPCC.

3.4.1.2 Familial adenomatous polyposis coli (FAP)

FAP is the most common polyposis syndrome, with a frequency varying from 1 in 10 000 to 1 in 30 000 persons. The genetic defect in FAP is a germ-line mutation in the APC gene. FAP is characterized by the occurrence of >100 polyps (adenomas) in the colon and rectum, although the number of polyps is often much higher and they usually become visible at before age twenty. The life-long risk for CRC is practically 100% and the average age of onset of CRC is 40 years without prophylactic colectomy (231). FAP patients also have a risk of other tumors. Diffuse mesenteric fibromatosis, termed desmoid tumor, occurs in 20–30% of patients, often after colectomy and this benign tumor may cause premature death due to abdominal and retroperitoneal complications related to the growth of the desmoid (232). FAP patients may also have duodenal adenomas which sometimes show malignant transformation and therefore upper-gastrointestinal endoscopy is included in the surveillance protocol (231). Other rare tumors associated with FAP are hepatoblastoma, thyroid cancer, brain tumors (Turcot syndrome) and gastric and pancreatic cancer.

3.4.1.3 Juvenile polyposis (JP) and Peutz–Jeghers syndrome (PJS)

Juvenile polyposis (JP) and Peutz–Jeghers syndrome (PJS) are two other gastrointestinal-polyposis syndromes predisposing to CRC. They are rare (1 in 100 000) and can be diagnosed on the basis of special histological features of the polyps (hamartomas). Germ-line mutation in the LKB1 gene predisposes for PJS and mutations in two different genes (SMAD4 or BMPR1A) for JP (233).

3.4.1.4 MUTYH-associated adenomatous polyposis (MAP)

A subset (about 15%) of APC mutation-negative patients with no autosomal dominant inheritance display adenomatous polyps ranging from 15 to 100 (234).
The MYH gene on human chromosome 1p33-34 is a base excision repair gene in which germline mutations have been found in association with multiple colorectal adenomatous polyps (235). These mutations may be missense or nonsense, the latter yielding protein truncation (236).

3.4.2 Familial CRC:

Ten to fifteen percent of patients with colorectal carcinoma and/or colorectal adenomas have other affected family members but their family histories do not fit the criteria for either FAP or HNPCC and may not appear to follow a recognizable pattern of inheritance, such as autosomal dominant inheritance. Such families are categorized as having familial colorectal carcinoma. The presence of colorectal carcinoma in more than

one family member may be due to genetic factors, shared environmental risk factors or even to chance (237).

A positive family history of CRC increases the estimated risk for CRC by two to four-fold. This means that life-long risk of CRC is between 10% and 20% in persons with a positive family history if they are living in a country with a high incidence of CRC. The enrichment of low penetrance susceptibility genes has been suggested as an explanation for the increased risk, but very little evidence has been obtained as yet (233).

3.4.3 Sporadic CRC

Approximately 75% of all new cases of colorectal carcinoma occur in people with no known predisposing factors for the disease. Incidence increases with age, beginning around 40 years (238). People with no predisposing factors are considered to be at average risk for colorectal carcinoma.

CRC develops through different pathways. The common mechanism (more than 50% of sporadic CRCs) is that of chromosomal instability (CIN) (239-241). The second pathway (35% of sporadic CRCs) is caused by epigenetic inactivation of tumor suppressor genes. Because of the mechanism involved in this, it is called the CpG island methylator phenotype (CIMP). A third pathway is caused by failure of the DNA mismatch repair system, and these tumors have a characteristic signature mutation called microsatellite instability (MSI), MSI-high (MSI-H), MSI-low (MSI-L) or MSI-stable (MSS) (242).

4 Screening methods for Colorectal Cancer:

The goal of colorectal cancer screening is to identify early cancers and adenomatous polyps by mass screening of all average-risk adults 50 years and older (243).

An average-risk adult is defined as an asymptomatic person without a personal or family history of adenomatous polyps or other illness (e.g., IBD, FAP, HNPCC) that predisposes to colorectal cancer. Persons at increased risk require more intensive screening(244).

Canadian Task Force on Preventive Health Care, 2001 (245)
• Good evidence to support annual or biennial fecal occult blood test (FOBT).
• Fair evidence to include flexible sigmoidoscopy in the periodic health examination of patients aged 50 years or older.
• Insufficient evidence to include or exclude colonoscopy as an initial screening test.
National Committee on Colorectal Cancer Screening, 2002(246)
• Recommends biennial (at minimum) FOBT for average-risk people aged 50 to 74.
• Recommends follow-up of positive FOBT by colonoscopy.
• Recommends screening occur in organized provincial programs, with ongoing evaluation.

Table (2) **Screening Guidelines for Canadians at Average Risk for Colorectal Cancer**

In Canada, guidelines for colorectal cancer screening recommend an FOBT every two years for people aged 50–74 who are at average risk for the disease. It is estimated that if 80% of Canadians within this age range had a biennial FOBT with appropriate follow-up through organized screening, alongside any opportunistic testing that occurs outside of organized programs, 10,000 to 15,000 colorectal cancer

deaths could be avoided over the next 10 years (247;248).

The National Colorectal Cancer Screening Network was launched by the Canadian Partnership Against Cancer in late 2007 to support the development of evidence-based screening programs and policy. As of the fall 2010, several organized colorectal cancer screening programs were established across Canada, with eight provinces currently running full or pilot programs and two provinces having announced intentions for a program. Each program follows the recommendations for colorectal cancer screening set out in the population-based guidelines developed in 2002. All programs are using some variation of the FOBT as the entry test—either the guaiac fecal occult blood test (gFOBT) or the fecal immunochemical test (FIT).

4.1 Structural examinations

4.1.1 Flexible sigmoidoscopy (FSIG)

The examination uses a sigmoidoscope, a flexible, lighted tube about the thickness of a finger with a small video camera at the end, so the images are displayed on a monitor. As the sigmoidoscope is only 60 cm long, it can see the entire rectum but less than half of the colon with the procedure. In addition, the examination can be done with different endoscopic instruments, such as a colonoscopy, an upper endoscope, and a pediatric colonoscopy. The main advantage of FSIG is that it can be performed with a simple preparation (2 Fleet enemas). However, bowel cleansing is best achieved through an oral sodium phosphate procedure. Patients had a more favourable experience with the oral preparation than with enemas (249). The examination usually lasts 10-20 min. The

absence of sedation is perceived by some patients as a benefit and by others as a disadvantage. Often, patients undergoing sigmoidoscopy relate more discomfort than patients undergoing colonoscopy. Moreover, the lack of sedation is related to greater reluctance of patients to undergo an examination for future screening.

4.1.2 Colonoscopy

The examination assesses the entire length of the colon and rectum with a colonoscope, which is basically a longer version of a sigmoidoscope. The colonoscope has a camera on the end that is connected to a monitor so we can see and closely examine the inside of the colon. The modern colonoscope can examine the entire intestine, with the examination terminating in a cecum. Patients typically take a liquid diet one or more days before the examination, followed by oral ingestion of lavender or saline laxatives to stimulate intestinal movements until the intestine is clean. Proper bowel preparation is a critical element in accuracy and cost-effectiveness of screening with colonoscopy (250). It is common for patients to receive a mild sedative before the procedure, but it is not essential for those who tolerate the procedure with only mild discomfort(251).

The test usually takes about 30 min, although it may take longer if a polyp is found and removed. The examination allows direct mucosal inspection of the entire colon and possibly a biopsy sample or even a definitive treatment such as polypectomy. The colonoscope is lubricated so that it can be easily inserted into the rectum. Once in the rectum, the colonoscope goes through the transverse colon and ascending colon. The colonoscope will deliver air into the colon so that it is easier to see the lining of the colon and to use the tools to perform the test. If a small polyp is found, it can be removed. This

is done by a wire running through the circuit colonoscope to cut the polyp from the wall of the colon with an electric current. In the case of a large polyp, cancer or anything else abnormal, a biopsy can be done. For this procedure, a small piece of tissue is taken by the colonoscope. Moreover, the tissue must be examined under a microscope to determine whether it is a cancer, a benign (not cancerous) growth, or a result of inflammation (252).

4.1.3 CT colonography

CT colonography is an imaging-only examination and does not offer the therapeutic option of polypectomy that optical colonoscopy does; however, in several current CT colonography screening programs, same-day colonoscopy after a positive CT colonographic study is being offered to eliminate a second bowel preparation for the patient (253).

CT colonography offers several advantages compared with other colorectal screening methods; it also has known limitations and risks. Advantages of CT colonography include that it is a minimally invasive, whole-colon structural examination with high sensitivity for detection of advanced neoplasia. The test is time efficient, typically taking 10 minutes at CT, which includes placement of a rectal tube with bowel insufflation. No sedation, recovery time, or need for a scheduled driver after the procedure is required, and patients have the capability to return to work the same day (254).

4.2 Stool-based markers

4.2.1 1-Fecal Occult Blood Testing

Fecal occult blood testing (FOBT) is the most widely used screening test for CRC. Two main types of FOBT exist, the guaiac test (g-FOBT), which is based on the peroxidase like activity of haem in haemoglobin, and the immunochemical test (i-FOBT), which detects the globin moiety in haemoglobin. Of these two, the guaiac test has been the more widely evaluated. It is a non-invasive simple test that requires no patient preparation but has a low sensitivity for CRC detection (40 - 85%). Four randomized trials have shown that screening with the guaiac based FOBT reduced both the incidence by approximately 20% and mortality of CRC by about 16% (255).

A study comparing i-FOBT to g-FOBT and colonoscopy as a gold standard found that for invasive cancers, sensitivity of i-FOBT is one and a half times more than g-FOBT and, sensitivity of i-FOBT is 3.3 times higher than g-FOBT for high risk adenomas. This demonstrates that i-FOBT outperforms g-FOBT and the increase in sensitivity for the detection of high risk adenomas is significantly greater than that of invasive cancers which makes it suitable for detection of patients at earlier stages (256).

4.2.2 Fecal DNA-based tests

Fecal DNA tests detect mutant or abnormal DNA shed from neoplastic colorectal lesions that is excreted in the stool. Since no single gene has been identified that is altered in all CRCs, a panel of DNA markers is usually employed. The most frequently measured

markers in stool include mutant K-ras, mutant APC, mutant P 53, BAT-26 and long DNA (257).

Studies show that DNA marker panels have a specificity of 95% or greater. However, sensitivity varied from 60% to 90%. To compare the use of specific DNA panels to FOBT, a large population based study was conducted. DNA panel detected 16 out of 31 invasive cancers, whereas FOBT detected only four. Of the 71 invasive cancers and adenomas diagnosed with high-grade dysplasia, the DNA panel detected 29 while FOBT detected only ten. In subjects with negative findings on colonoscopy, the DNA panel had a specificity of 94.4% and FOBT had a specificity of 95.2%. Although neither techniques detected the majority of neoplastic lesions, the DNA panel displayed a higher sensitivity than FOBT without reduced specificity (258-260).

Despite the better performance of the DNA panel as compared with FOBT, its overall low sensitivity in asymptomatic subjects, coupled with relatively high cost and assay complexity makes it unlikely that molecular markers will replace the FOBT as a widely used screening tests for CRC.

5 Diagnosis and treatment of CRC

Prognostic markers are defined as markers which can identify patients with differing risks of a specific outcome, such as tumor progression or death but are not used to guide the choice of a particular therapy. A predictive marker is one that predicts the differential efficacy or benefit of a particular therapy based on marker status and therefore could be used to guide the choice of therapy (261).

5.1 Tumor node metastasis (TNM) staging:

TNM stage remains the gold standard of prognostic factors in CRC. The TNM staging was initially developed to predict prognosis, but its function has expanded to aid in the choice of treatment and in the selection of patients for clinical trials.

The stage often takes into account the size of a tumor, how deeply it has penetrated, whether it has invaded adjacent organs, how many lymph nodes it has metastasized to (if any), and whether it has spread to distant organs (262).

TNM Staging System (Tumor, Node, Metastasis)

Tumor

T1: Tumor invades submucosa.

T2: Tumor invades muscularis propria.

T3: Tumor invades through the muscularis propria into the subserosa, or into the pericolic or perirectal tissues.

T4: Tumor directly invades other organs or structures, and/or perforates.

Node

N0: No regional lymph node metastasis.

N1: Metastasis in one to three regional lymph nodes.

N2: Metastasis in four or more regional lymph nodes.

Metastasis

M0: No distant metastasis.

M1: Distant metastasis present.

Stage Groupings

Stage I: T1 N0 M0; T2 N0 M0

Cancer has begun to spread, but is still in the inner lining.

Stage II: T3 N0 M0; T4 N0 M0

Cancer has spread to other organs near the colon or rectum. It has not reached lymph nodes.

Stage III: any T, N1-2, M0

Cancer has spread to lymph nodes, but has not been carried to distant parts of the body.

Stage IV: any T, any N, M1

Cancer has been carried through the lymph system to distant parts of the body. This is known as metastasis. The most likely organs to experience metastasis from colorectal cancer are the lungs and liver.

Most colorectal cancer patients present with symptoms such as bleeding, obstruction or abdominal pain, although increasing numbers of cases are identified through screening. The diagnosis of colorectal cancer is commonly made using colonoscopy or sigmoidoscopy, which enables physicians to identify the exact location of the tumour and perform a biopsy. A barium enema and computed tomography (CT)

scanning may also be used. Following diagnosis, staging tests such as CT scans are frequently performed.

The treatment of stage I, II and III colorectal carcinoma typically involves surgery:

_ For some stage II and for stage III colon cancer, chemotherapy after surgery is frequently used.

_ For stage II and stage III rectal cancer, a combination of chemotherapy and radiation is often provided, preferably before surgery.

_ Stage IV colorectal cancer, where there is spread to sites away from the primary tumour, is typically treated with palliative chemotherapy with surgery and/or radiation used predominately for symptoms. A small proportion of people with stage IV colorectal cancer can have long-term survival with aggressive surgery and chemotherapy (263).

6 Tumor markers

6.1 Definition and clinical use

Most researchers in this field prefer defining tumor markers as: a molecule, a process, or a substance that is altered quantitatively or qualitatively in precancerous or cancerous conditions, the alteration being detectable by an assay. Alterations can be produced either by the tumor itself or by the surrounding normal tissue as a response to tumor cells. The tumor marker itself can be DNA, mRNA, protein, or processes (apoptosis, angiogenesis, proliferation and so on) measured quantitatively or qualitatively by an appropriate assay (264).

There are many possible clinical uses of tumor markers and several categories have been defined. **A diagnostic tumor marker** is a marker that will aid in detection of malignant disease in an individual, if the marker is to be used for mass screening; a fundamental prerequisite is to exhibit both high levels of diagnostic sensitivity and specificity (265). Meanwhile, **a prognostic marker** gives the clinician a tool for estimating the risk of disease recurrence and/or cancer-related death for an individual patient following the initial surgical removal of the cancer but without administration of adjuvant therapy. In contrast, **a predictive tumor marker** will predict how the patient is going to respond to a given therapy (266). Many markers may have both a prognostic and a predictive value, an example of this is the Estrogen receptor (ER) content on breast tumours which plays two important roles: First, ER is a prognostic marker, in that ER-negative tumours are associated with greater failure hazard; and second, ER is a

predictive marker for response to anti-estrogen drugs. Virtually no response is noted in patients with ER-negative tumours, while those with ER-positive tumours respond (267).

6.2 Quality Requirements for the Use of Tumour Markers in Clinical practice:

Diagnostic test accuracy refers to the ability of a test to discriminate between those who have and those who do not have the target condition. Accuracy is assessed by the results of the index test, the test under evaluation, with the results of the reference standard, which aims to classify patients as having or not having the target condition. The aim of diagnostic test evaluation is to address clinical questions such as "Should the patient undergo this diagnostic test?" and after ordering the test and seeing the test result, "What is the likelihood that this patient has the disease.

6.2.1 Defining Diagnostic Test Accuracy

6.2.1.1 Sensitivity and Specificity

Sensitivity is the probability of a positive test result (that is, the test indicates the presence of disease) for a patient with the disease. Specificity, on the other hand, is the probability of a negative test result for a patient without the disease(that is, the test does not indicate the presence of disease).

True-positives (TPs) are those patients with the disease who test positive. True-negatives

(TNs) are those without the disease who test negative. False-negatives (FNs) are those with the disease but the test falsely indicates the disease is not present. False-positives (FPs) are those without the disease but the test falsely indicates the presence of disease. Sensitivity, then is the probability of a TP among patients with the disease (TPs + FNs). Specificity, is the probability of a TN among patients without the disease (TNs + FPs).

6.2.1.2 Positive and Negative Predictive Value:

In clinical practice it is essential to know how a particular test result predicts the risk of abnormality. Sensitivities and specificities do not do this: they describe how abnormality (or normality) predicts particular test results. Predictive values do give probabilities of abnormality for particular test results, but depend on the prevalence of abnormality in the study sample and can rarely be generalized beyond the study (except when the study is based on a suitable random sample, as is sometimes the case for population screening studies) (268).

An important question for the clinician to decide on the proper management for patients is "What is the likelihood that this patient has the disease when the test result is positive?" and "What is the likelihood that this patient does not have the disease when the test result is negative?"

The positive predictive value (PPV) is the probability that the subject has the disease when the test is positive.

PPV=TP/ (TP + FP) (269).

The PPV differs from sensitivity. While the PPV tells us the probability of a subject having the disease following a positive test (e.g., the probability of a subject having colorectal cancer after testing positive with GTA-446), the sensitivity tells us the probability that the test will be positive among subjects with the disease (e.g., the probability of testing positive for GTA-446 among colorectal cancer patients). PPV helps the clinicians decide how to treat the patient after the diagnostic test comes back positive. Sensitivity on the other hand is a feature of the diagnostic test and helps the clinician decide which test to use.

The Negative Predictive Value (NPV) is the probability that the disease will not be present when the test is negative.

NPV=TN/(TN + FN) (270)

The NPV is different from the test's specificity. Specificity tells us the probability that the test will be negative among subjects without the disease (270) (e.g., the probability of negative GTA-446 test among subjects without colorectal cancer).

6.2.1.3 Likelihood ratio:

Each test result has its own likelihood ratio, which summarizes how many times more (or less) likely patients with the disease are to have that particular result than patients without the disease. More formally, it is the ratio of the probability of the specific test result in people who do have the disease to the probability in people who do not(271).

A likelihood ratio greater than 1 indicates that the test result is associated with the presence of the disease, whereas a likelihood ratio less than 1 indicates that the test result is associated with the absence of disease. The further likelihood ratios are from 1, the stronger the evidence for the presence or absence of disease. Likelihood ratios above 10 and below 0.1 are considered to provide strong evidence to rule in or rule out diagnoses respectively in most circumstances. When tests report results as being either positive or negative the two likelihood ratios are called the positive likelihood ratio and the negative likelihood ratio.

Positive Likelihood Ratio= Sensitivity / 1-Specificity

Negative Likelihood Ratio= 1-Sensitivity / Specificity (272)

The likelihood ratio of a positive test result reflects the amount of certainty of having the disease that is gained after a positive test result, whereas the likelihood ratio of a negative test result is the amount of certainty gained of not having the disease with a negative test result(271).

For example. A positive likelihood ratio of five is interpreted as 'in patients with the disease, a positive test is found five times as often as in patients without the disease.' The negative likelihood ratio on the other hand is the amount of information that is gained after a negative test result.

A likelihood ratio close to 1 indicates that performing the test provides little additional information regarding the presence or absence of the disease. The likelihood ratios have the advantage of putting equal weights to the sensitivity and specificity and therefore being less dependent on the proportion of individuals under study who are diseased versus non-diseased (273).

6.2.1.4 Diagnostic Odds ratio (DOR)

The DOR is equivalent to the ratio of positive and negative likelihood ratio

DOR = Positive likelihood ratio / Negative likelihood ratio

The value of a DOR ranges from 0 to infinity. A value of one means that a test does not discriminate between patients with the disorder and those without it.

The advantage of the DOR is that it summarizes in one figure the diagnostic association between the test and the disease (274).

An important notion is that odds ratios do not characterize the discriminatory capacity of a marker. The odds ratio is a simple scalar measure of association between marker and outcome. It does not characterize the discrimination between cases and controls that can be achieved by a marker since many different pairs of sensitivities and specificities are consistent with a particular odds ratio value (273;275). For example, for a marker with a sensitivity of 80% and specificity of 90% the odds ratio is huge: 36. However, that even for an odds ratio as large as 36, one cannot conclude that the marker has good accuracy since a variety of (Sensitivity, Specificity) values are consistent with it (e.g., specificity = 0.50, sensitivity = 0.973) also yields an odds ratio of 36.

6.2.1.5 Receiver operating characteristic curve (ROC);

ROC is a plot of a test's false positive rate (FPR) or 1- specificity (plotted on the horizontal axis), versus its sensitivity (plotted in the vertical axis). Each point on the

curve represents the sensitivity and FPR at a different decision threshold. The plotted (FPR, sensitivity) coordinates are connected with line segments to construct an ROC curve. In an ROC curve, every possible decision threshold is considered (276).

An ROC curve begins at the (0,0) coordinate, corresponding to the strictest decision threshold whereby all test results are negative for disease. The ROC curve ends at the (1, 1) coordinate, corresponding to the decision threshold whereby all test results are positive for disease. The line connecting the (0, 0) and (1, 1) coordinates is called the "chance diagonal" and represents the ROC curve of a diagnostic test with no ability to distinguish patients with versus those without disease. An ROC curve that lies above the chance diagonal has some diagnostic ability. The closer the ROC curve to the upper left hand corner, the better discriminating power and diagnostic accuracy the test has (277).

The ROC curve of the test provides much more information about how the test performs than just a single estimate of the test's sensitivity and specificity. Given a test's ROC curve, a clinician can examine the trade-offs in sensitivity versus specificity for various decision thresholds. Based on the relative costs of false positive and false negative errors and pretest probability of disease, the clinician can choose the optimal decision threshold for each patient. One can derive the optimal cut-off from the relative importance of false positives and false negatives. For example, a missed (false negative) colorectal cancer is more serious than a false positive one (278).

A measure of performance for the test is area under the ROC curve (AUC), it ranges in value from 0.5 (chance) to 1.0 (perfect discrimination or accuracy).
AUC represents the probability that, when presented with a randomly chosen patient with disease and a randomly chosen patient without, the results of the diagnostic test will put

the patient with disease as having higher suspicion for disease than the patient without disease (279). For example, if a biomarker has an AUC of 0.8 for differentiating between disease and non disease, this means, that if two subjects were randomly chosen from disease and non disease groups and the biomarker was measured and the test results are used to guess which of the two is the diseased, the test will be right 80% of the time.

A key advantage for ROC is that ROC curve does not depend on how the marker is coded. Changing the units in which the marker is measured has no impact on its ROC. Moreover, ROC curves provide a natural common scale for comparing different markers even when they are measured in completely different units. In contrast, because odds ratios are interpreted per unit increase in the marker, odds ratios for two markers may not be comparable (278).

6.2.1.6 Logistic regression for many diagnostic tests

Logistic regression is a method for analysis of binary data, such as the presence or absence of disease.

For a single dichotomous test the logistic regression equation is:

$$P(D|x) = \frac{1}{1+\exp^{-(\alpha+\beta x)}}$$

where x stands for the test result and the coefficients have to be estimated.

If a positive test result is coded as x = 1 and a negative as x = 0, we have

$$P(D|positive) = \frac{1}{1+\exp^{-(\alpha+\beta x)}}$$

which is the probability of having the disease given the test (x) is positive

and

$$P(D|\text{negative}) = \frac{1}{1 + \exp^{-\alpha}}$$

which is the probability of having the disease given the test (x) is negative.

Next, diagnostic Odds Ratio is expressed as:

$$DOR = [P(D|\text{positive}) / (1-P(D|\text{positive}))] / [P(D|\text{negative}) / (1-P(D|\text{negative}))] = \exp(\beta).$$

In other words, the DOR equals the regression coefficient, after exponentiation (280). Logistic regression modeling has been proposed as the preferred statistical method to obtain a post-test probability of disease when results from multiple tests are available. History taking and physical examination can also be considered as individual diagnostic tests. The post test probability after having obtained test results xl,x2, ...xk is expressed as

$$P(D|X_1, X_2 \ldots X_k) = \frac{1}{1 + \exp^{-(\alpha + \beta_1 x_1 + \beta_2 x_2 + \ldots\ldots + \beta_k x_k)}}. \quad (281)$$

With multiple dichotomous tests of which the results x1, x2 ... xk are coded as present (1) or absent (0), the corresponding coefficients B_1, B_2,…B_k equal the conditional logDOR. These DOR's are conditional: they depend on the other variables that have been used in the model. If more information becomes available a new regression equation has to be constructed to obtain the proper conditional DOR (280;282).

6.2.1.7 Confidence Intervals:

It is important to assess how confident we can be about the test characteristics. If a test produces 80% sensitivity, it is unlikely that we will again find exactly 80% for sensitivity in a new series of patients, even when the same patients would have been retested in the same or another setting, different data will be obtained.

The statistical method for analyzing the variability in estimates of diagnostic accuracy measurements is the use of confidence interval. The probability level of the confidence interval can be chosen (283).

The interpretation of 95% confidence interval for an estimate is: when the data sampling is repeated many times, the 95% confidence interval calculated from each sample will, on average, contain the "true" value of the proportion in 95% of the samples (284).

6.3 Problems introduced as a result of specific population selection

The main objective to carry out a trial is to generalize the results beyond the study population itself. In order to do that, we need to know to what extent the patients we have studied are representative of all patients with the disease in question.

In the design of any clinical diagnostic study, a fundamental requirement is to use a proper reference standard to which the diagnostic test is to be compared (285).

6.3.1 Referral bias:

Referral bias occurs because people who are referred to participate in a study are often different from non-referrals (286). This could happen if a study is conducted in a large, specialized hospital that has a high concentration of rare, difficult or complex cases compared to real world population. The same effect could be obtained by physicians who voluntarily select these cases and refer them to participate in the study (285).

Another source of referral bias is introduced when patients with a positive (or negative) diagnostic test are preferentially selected to receive verification by the "gold standard" examination. In the case of positive test results, the patients selected for additional workup are more likely to have disease than those excluded and therefore are more likely to have a true positive result. Alternatively, patients with negative results may actually have disease that goes undetected because definitive testing was not performed. These cases would normally increase the number of false negative reports, but because they are not identified they are erroneously labeled as true negatives (287).

6.3.2 Population bias

Population bias is introduced when there is a big difference in disease prevalence in the studied sample in comparison with what really exists in real population (288).
A feature of PPV and NPV is their dependence on the prior probability of the disease (which is equal to the prevalence of disease in the population to be tested), i.e., if the disease prevalence increases, a positive test result will have a higher PPV. This is due to a relative decrease in the number of patients with a false-positive result to the number of

true positives. As a consequence, the proportion of true positives among the total number of those with a positive test result will rise, resulting in a higher PPV. The opposite will occur for NPV, that is higher disease prevalence will result in a lower NPV. Therefore, when comparing diagnostic accuracy measures between different populations, different results can be obtained. If the studied population has a high prevalence of the disease compared to the normal population, PPV will be erroneously overestimated and the assessment of the diagnostic test will be distorted (289).

6.3.3 Spectrum bias

When a diagnostic study starts by including patients who have already received a diagnosis of the target condition and uses a group of healthy volunteers as the control group, it is likely that both sensitivity and specificity will be higher than they would be in a study made up of patients only suspected of having the target condition. This feature has been described as spectrum bias (290).

If the enrolled patients have disease type, severity, or duration that are clearly different from those of patients commonly found in clinical practice, a bias happens.

In spectrum bias, mild cases that are difficult to diagnose are omitted from case-control studies, causing an overestimation of sensitivity as well as specificity (45).

Indices of test efficacy, such as sensitivity, specificity, and likelihood ratios, are often considered to be fixed properties of a test that do not vary as disease prevalence changes among population (291). However, different sensitivities and specificities could be obtained in women versus men, for elderly versus younger individuals and in patients with and without disease. For this reason, it is necessary to present strata-specific

estimates of the measures of disease accuracy for relevant subgroups. Also, the stage of disease severity could affect the sensitivity and specificity of a test.
This shows that tests may perform differently in different groups of subjects and for different severities of disease (292).

There are however particular situations where the study design purposefully includes spectrum bias. This is the case during the first attempts to evaluate the diagnostic performance for a given test. The first phase of evaluation of a new test is assessment of the test using a study including the "easily accessible population". If the test performs badly here, it will certainly perform worse in clinical practice. Conversely, if the new test was found to have sufficient discriminative power, we can go ahead with other larger studies. However, even in the event of a very good performance of the new test in the first phase, we cannot transfer these results to clinical practice. The reason is the presence of a huge spectrum bias (293).

After the initial experimental phase, if the purpose is to estimate the diagnostic performance of a new test to be applied in clinical practice, the solution of spectrum bias is to enroll a random sample of the population that could undergo the test modality in clinical practice. If the testing is for a screening method, a random sample of asymptomatic subjects with suitable demographics should be included (294).

7 HISTORY OF DISCOVERY OF GTA-446

Phenomenome Discoveries Inc. (PDI) located in Saskatoon was able to detect a novel biomarker in the serum using a non-targeted approach. This approach characterizes the metabolic profiles to identify novel serum metabolomes in CRC patients compared to healthy controls. An advanced technology using high resolution Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) was used to accurately identify the elemental composition of all ions detected in a sample.

7.1 Patient samples used for the discovery project

Three sets of samples were used in the discovery project, they were obtained from Genomics Collaborative Inc. (GCI), Seracare Life Sciences, and Osaka Medical Universities. GCI and Seracare were companies that specialize in the collection and storage of serum and tissue samples specifically for research purposes.

The total number of samples were 112 CRC patients and matched controls. All samples were taken prior to any attempted treatment for CRC and were accompanied by detailed pathology reports. All samples were stored at -80◦c until time of analysis.

7.2 Sample extraction protocol and technology used in discovery of the novel biomarkers

Liquid extraction was performed on all serum samples by adding an equal volume of serum to 1% ammonium hydroxide in ethylacetate followed by centrifugation and

transfer of the top organic layer into a new tube. This process was repeated three times with pooling of the organic extract into a new tube (extract A). A one to five ratio of extract A to butanol was then evaporated under nitrogen to the original butanol starting volume which is now ready to be analyzed by FTICR-MS.

7.2.1 FTICR-MS analysis

Mass spectrometry is an analytical instrument that converts components of a sample into gaseous ions and measures their mass. This measurement is useful in the identification of compounds introduced into the mass spectrometer. Although there are many types and designs of mass spectrometers, there are common features among them.

- All mass spectrometers ionize (charge) molecules (i.e. ion sources must be used to generate the gas-phase ions from a neutral sample) although the mechanisms for ionization vary markedly.
- Every mass spectrometer has a mechanism to separate ions based on their mass (m) and the number of charges (z) present. This portion of the instrument is called the "mass analyzer" and can be of various designs and configurations.
- All mass spectrometers have a detector that can detect the ions that reach it after having passed through the analyzer.

Mass to charge ratio (m/z). An abbreviation used to denote the quantity formed by dividing the mass of an ion by the number of charges carried by it, e.g. a singly charged ion has an m/z equal to its mass.

Nominal mass. This is the mass of an ion calculated by adding the integer masses of the lightest isotopes of all elements contributing to a molecule.

Resolution. This is the ability of a mass spectrometer to distinguish between ions of different m/z ratios. Greater resolution corresponds directly to the increased ability to differentiate ions of similar molecular weights (295).

7.2.1.1 Principles of FTICR-MS

Among all the different varieties of available mass spectrometers, FTICR-MS is considered a powerful instrument due to the inherently ultra-high resolution and mass accuracy which can be achieved. Separation of ions is based on their motion in a magnetic environment, which causes them to orbit at different frequencies, called cyclotron frequencies, depending on their m/z. Detection of the ions occurs as they pass two detector plates and generate an alternating electrical current . The magnitude of the signal is proportional to the total charge and the orbital radius (the proximity of the ions to the detection plates). The potential (voltage) change between the detection plates can be measured as a function of time. The frequency information is obtained from time-domain data through a mathematical procedure known as “Fourier transform”. A second mathematical operation converts the frequency spectrum to a mass spectrum (296).

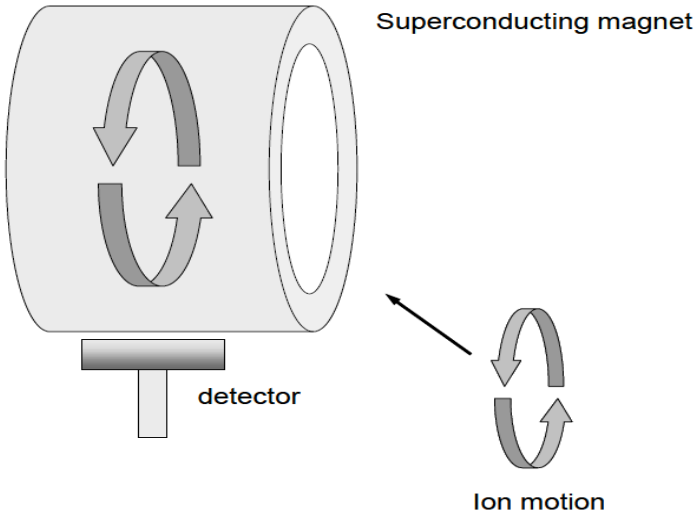

Figure (4) **Simplified illustration of an FTICR-MS**. Ions enter a circular path at different frequencies. These frequencies of rotation are detected and transformed mathematically into a mass value (295).

7.2.1.2 Sample injection and data acquisition

Extracted samples were directly injected at a flow rate of 600 uL/hour into FTICR-MS, and the instrument was calibrated such that each internal standard mass peak has a mass error of < 1 part per million compared with its theoretical mass.

The compounds in the range of 100 -1000 m/z were analyzed and the mass spectra from each analysis were integrated and combined so as to create one data file per sample that contain all the unique masses. These data were then merged and extrapolated to create a two-dimensional metabolite array in which each sample is represented by column, and each unique metabolite is represented by a single row.

7.3 Results of data analysis

Examination of the data showed an area of reduced peak intensities in the region of 440 and 600 Da in CRC patients relative to controls that was statistically significant. This was followed by ranking the top 50 masses based upon probability value from each of the three independent studies. Filtering for metabolic differences detected in all three studies resulted in 13 masses that represent the most statistically significant and robust discriminators among the three studies.

Computational calculation of the molecular formulae were then carried out for the 13 masses to determine the number of carbons, hydorgens, oxygens, and other elements based on their exact mass. This resulted in the conclusion that the compounds are composed of either 28, 30, 32, or 36 carbons and four to six oxygen atoms.

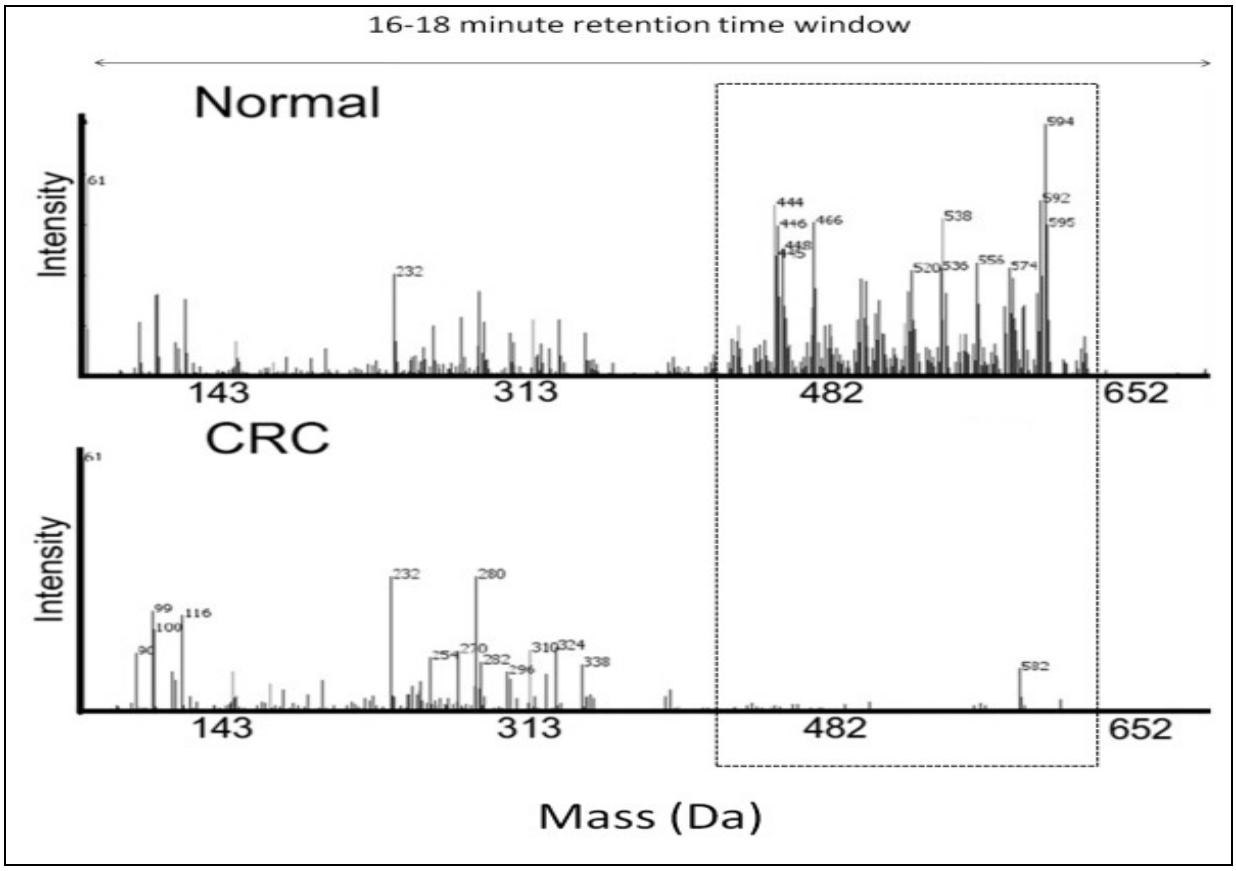

Figure (5)**Extracted mass spectrum of serum from normal subjects and colorectal cancer (CRC) patients.** Extracts from five representative CRC and five control samples from the Genomics Collaborative discovery set were subject to high performance liquid chromatography followed by full-scan detection on an Applied Biosystems QSTAR XL™ mass spectrometer in atmospheric pressure chemical ionization negative mode. The average intensities of all ions within the mass range 100 to 700 Da eluting between 16 and 18 min are shown for each cohort. The boxed region indicates spectral features present in normal patients but absent from CRC-positive serum (297).

7.4 Structural elucidation

Tandem mass spectrometry was used to identify the structure of the new molecules. Tandem mass spectrometry employs two series of mass analysis, the two steps

of mass analysis can be separated in either space (e.g. triple quadrupule) or time (e.g. ion traps). Most commonly, the first mass analyzer (MS1) isolates a single m/z value, called the "precursor ion". Between MS1 and the second mass analyzer (MS2), ions are caused to dissociate into fragments, or "product ions" in a chamber called a "collision cell". The fragmentation pattern of the precursor/product ion mass pair (called MS/MS transition) is exceptionally unique and highly selective, because two different ions (even with the same m/z) are unlikely to have the same precursor ion mass and product ion mass (295).

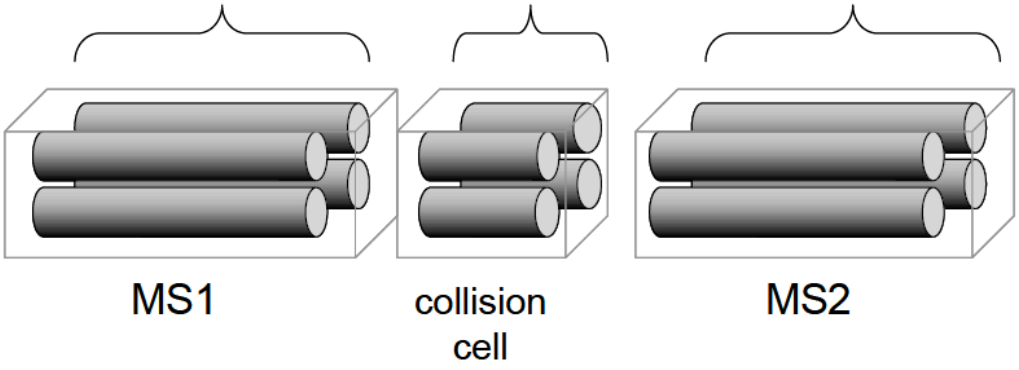

Figure (6) **Tandem MS illustrating all three quadrupole regions.** The first is mass analyzer MS1; the second is the collision cell; and the third is the mass analyzer MS2, where the products of the collision cells are separated (295).

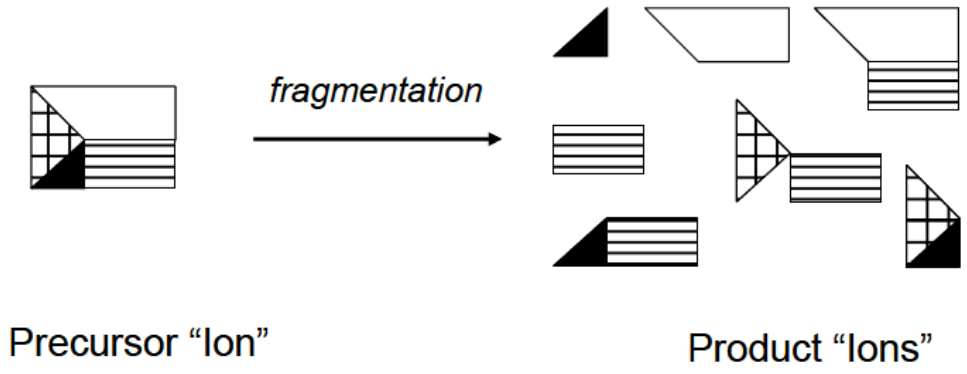

Figure (7) **An illustration of the fragmentation of a generic molecule.** The precursor ions fragment in a reproducible way, forming specific product ions. The product ions formed are based on the structure of the precursor ion (and thus the precursor molecules) (295).

One of the common uses of the Tandem MS is chemical structure characterization. This helped shed some light on the structure of the novel biomarkers that were detected by FTICR-MS. Analysis of the Tandem mass spectrometric fragmentation fingerprints for the C28 molecules revealed main losses of H2O and CO2, indicating the presence of a carboxylic acid group and two or more hydroxyl groups. This prompted the hypothesis that these compounds could be derived from the same chemical family of fat soluble vitamins (Vitamins A, D, E, and K), steroids, bile acids, or long chain polyunsaturated hydroxy fatty acids. Fragmentation patterns of standards of these compounds were compared against C28 molecules, none of them were similar to the C28

biomarker except for the hydroxyl fatty acid standards which showed peripheral and chain cut ions[1] similar to those produced by MS/MS of the C28 molecules.

7.5 Key clinical findings of the preliminary studies

Three different sets of samples comprising three independent studies were used in the discovery project of the new biomarkers (GTA). All three studies showed a consistent reduction of these biomarkers among CRC patients compared to controls and ROC curve analysis resulted in an average area under-the-curve (AUC) of 0.91±0.03 across all three studies combined.

In addition, the authors did not observe differences between genders in terms of GTA biomarker levels in the blood, neither did they observe any correlation between the reduction of metabolites and disease stage. Next, the level of the GTA markers were measured in 990 serum samples collected at SDCL along with age and gender data, results showed reduction of the level of these markers with increasing age (297).

Finally, a study following up patients before and after colorectal cancer treatment revealed no restoration of the reduced level of GTA biomarkers post-treatment.

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[1] This terminology is specific to fatty acid fragmentation. MS/MS ions are clustered into three types: "peripheral-cut" ions, formed by neutral loss of water, CO_2, amino acid, or amines derived from functional groups linking to the LM carbon chain as hydroxy, hydroperoxy, carbonyl, epoxy, carboxy, amino acid group, or amino group; "chain-cut" ions, formed by the cleavage of a carbon-carbon bond along the LM carbon chain; and "chain-plus-peripheral-cut" ions, formed by a combination of chain cut and peripheral cut. Molecular ions formed during ESI can easily be converted to peripheral-cut ions in the MS/MS process. Similarly, chain-cut ions can also be readily converted to chain-plus-peripheral-cut ions (298).

Treatment groups included those who had surgery, those who did not have surgery, and patients that did have surgery who subsequently had chemotherapy, or combination of chemo/radiotherapy. The authors concluded that these metabolites are not "tumour-derived markers" and that the reduction is not likely the result of tumour burden.

7.6 The proposed role of the new GTA biomarkers

Structural elucidation of the new biomarkers showed them to be chemically related to long chain hydroxylated fatty acids. This directed the attention towards other known hydroxylated long chain fatty acids such as the resolvins and protectins that have a role in the resolution of acute inflammation (299).

A hypothesis was generated that the new GTA biomarkers have anti-inflammatory properties and that reduction of these metabolites with age may result in a state of chronic inflammation that has been linked to cancer.

An experimental in-vitro study was carried out to test the effect of chromatographically separated GTA-rich serum on different cell lines and on inflammation induced cell lines compared to GTA-deficient serum. The GTA rich fraction showed a 40% reduction in cell viability evidenced by the detection of apoptosis markers such as NF-KB and Nitric oxide synthase 2. The results showed that serum extracts containing GTAs have anti-proliferative properties compared to GTA deficient extracts which seem to lack these properties (300).

8 SUBJECTS AND METHODS

8.1 Objectives and Hypothesis

The purpose of this non-randomized, multi-center study is to assess the efficacy of a novel serum based biomarker screening test, (Gastric tumor acid, MW=446, or GTA-446 test), for colorectal cancer as compared against colonoscopy and pathology results. The effectiveness of the test will be defined by sensitivity (ability to predict CRC positive cases) and specificity (ability to predict CRC negative cases) under usual clinical care conditions.

Specific Objectives

1. Determine the correlation between GTA-446 levels and colon pathology.
2. Comparative results of the above biomarker data with histological finds from pathology samples
    - No neoplasia
    - Polyps with high risk of malignancy.
    - Polyps with low risk of malignancy.
    - Cancer
    - Other conditions affecting the colon such as: ulcerative colitis and Crohn's disease.
3. Validation of Saskatchewan Disease Control Laboratory samples via replicate analysis at PDI.

8.2 Study design

Between June of 2008 and August 2010, 4924 subjects scheduled for colonoscopy were enrolled in the study at two endoscopy centers; Regina General and Pasqua Hospitals in Regina, Saskatchewan.

After obtaining a written informed consent, a study nurse collected subject's demographic information (including age, sex), current medical history (including liver, gastrointestinal diseases and cancer), family history of cancer and pathology reports of the performed colonoscopies. Also in the same visit, a blood sample (~10 mL) was collected, centrifuged and split into two tubes.

The serum was sent frozen to Saskatchewan Disease Control Laboratory (SDCL) and the replicate samples were sent to Phenomenome Discoveries Inc.(PDI) after being depersonalized by removing all the identifying information and assigning an accession number. All the samples were blindly extracted and analyzed at SDCL. The corresponding clinical results were only released after completion of the analysis and release of the results.

8.2.1 Study population

Cases. Subjects consisted of males and females from age 18 to 80 years who were scheduled for colonoscopy at Regina General Hospital or Pasqual Hospital for any reason.

Controls. Two sets of controls were used for the study; First were the Lab controls (N=383) which were serum samples collected from SDCL along with age and gender data,

Second were the Endoscopy controls (N=762) which were obtained from the colonoscopy population after being determined to be colonoscopy free and had no risk factors for developing colorectal cancer compared to the average risk population.

8.2.2 Analytical method and mass spectrometry analysis

8.2.2.1 Extraction of serum samples:

Serum samples were stored at -70˚C until time of analysis, sample preparation involved addition of 15.4 uL of 10ug/mL [$^{13}C_1$] cholic acid to 0.4 mL of serum. This was followed by a 4-step liquid-liquid extraction by the addition of 0.4 mL of serum with 5 ml ethylacetate/1% ammonia solution followed by 5 ml ethylacetate/4% formic acid. Samples were shaken for 10 minutes and centrifuged at 3500 rpm between extractions; the organic layer was transferred and pooled to a new tube.

A hundred uL of the extracted samples were injected by flow injection and analyzed on an API-4000 triple quadrupole mass spectrometer using negative atmospheric pressure chemical ionization. Multiple Reaction Monitoring (MRM) was used to detect and calculate raw peak areas for gamma tocoenoic acid (GTA-446) C28H46O4 (MW 446, 445.3/383.4 [M-H] Da) and [$^{13}C_1$] cholic acid (MW 409, 408.3/343.4 [M-H] Da). Qualitative and quantitative MRM were recorded for each analyte. After the raw data was acquired, the peak areas for all samples were calculated using the Intelliquant^{TM} method in the Quantitation Wizard module of the Analyst^{TM} instrument software.

8.2.2.2 Calibration curve and quality control:

Calibration curve was prepared using serial dilutions of System Suitability Standard (SSS) which is ethyl acetate extract from healthy representative serum. SSS

serum was extracted and aliquoted by PDI so that the material used for the calibration curve is the same at both labs.

SSS was diluted with ethylacetate to prepare the following SSS dilutions; 1, 0.5, 0.25, 0.1, 0.05 and 0.01.

Quality control material was prepared using aliquoted pooled serum that was kept at -70˚c until analyzed.

On each analytical day, a single calibration curve was obtained and the quality control (QC) samples were analyzed. Intra-assay precision was calculated as 7% and inter-assay precision was 13% for the whole study.

8.2.2.3 Calculation of the results

Two methods were used to estimate the concentration of GTA-446 in serum samples; the first method was used for analysis of the samples in SDCL, it was done by dividing the raw peak area of GTA-446 in study samples by that of the SSS so that all results will be represented as a ratio level and expressed as SSS equivalent.

The second method was used for calculation of the results at PDI (the method was developed later after analysis of samples has already started in SDCL). It involved the use of [$^{13}C_1$] cholic acid equivalents (CAE) as a representative of GTA-446 concentration which is calculated by extrapolation from a [$^{13}C_1$] cholic acid standard curve.

9 Statistical methods:

Statistical analysis was performed using SPSS for windows version 19 & 20.

Plotting of ROC curve was performed using SPSS and MedCalc softwares.

10 RESULTS

10.1 Patient characteristics

Serum samples from 4924 subjects who underwent colonoscopy were collected along with pathology results, personal, past and family history. This allowed the categorization of colonoscopy population into four groups (Figure 8 and Table 3)

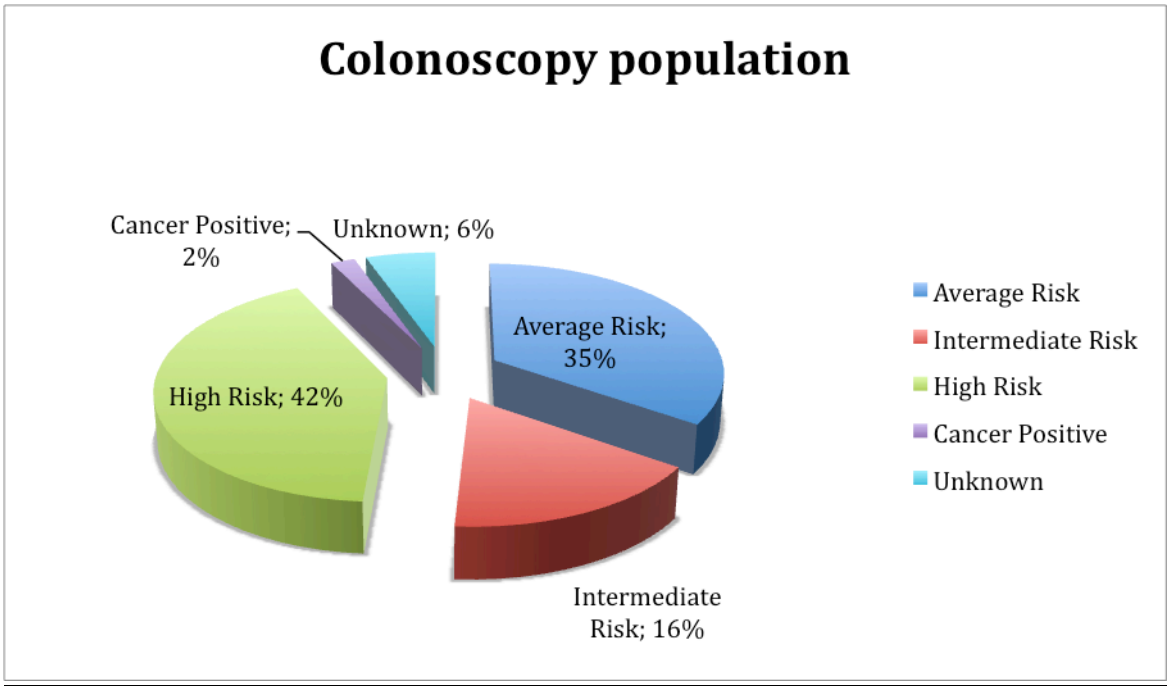

Figure (8) **Breakdown of the colonoscopy population into four groups.**

Subjects with average risk for colorectal cancer (N=1706) had to fulfill all of the following criteria: normal colonoscopy outcome (i.e. no polyps, inflammatory bowel disease or any other type of pathology), hereditary risk of CRC[2] is determined to be average and no past or family history of CRC (302).

Subjects whom colonoscopy results showed less than or equal to two polyps of benign nature, have a single 2nd degree relative CRC or have past history of uterine or ovarian or breast cancer were classified as intermediate risk (N=811) (303).

Subjects were considered at high risk to develop CRC (N=2042) if at least one of the following criteria existed: pathology shows polyps of advanced grade of dysplasia, presence of inflammatory bowel disease, positive past history of CRC, positive family history of a first degree relative, or more than two 2nd degree relatives with CRC. The fourth group includes subjects confirmed by colonoscopy to have colorectal cancer (N=94). Two hundred and seventy one cases were not classified into one of the four groups due to missing one or more aspects of the clinical data.

[2]Hereditary CRC means it is mendelian in nature- that is inherited in an autosomal dominant manner. The two major forms of hereditary CRC are FAB and HNPCC(304).

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[2] Hereditary CRC means it is mendelian in nature- that is inherited in an autosomal dominant manner. The two major forms of hereditary CRC are FAB and HNPCC(301).

	Average Risk N=1706		Intermediate Risk N=811		High Risk N=2042		Cancer Positive N=94	
	Males	Females	Males	Females	Males	Females	Males	Females
Number	728	978	370	441	928	1114	63	31
Age in years(mean/Range)	55 (18-89)		57 (20-89)		58 (20-92)		65 (28-89)	
Positive for Polyps	0		620		793		0	
Positive for cancer	0		0		0		94	
Hereditary CRC risk	0		0		1223		19	
Past history of CRC	0		0		199		22	
Family history of CRC	0		191		1416		23	
Inflammatory bowel disease (ulcerative colitis and crohn's disease)	0		0		220		1	
Total	4653 / 4924							

Table (3) **Subject characteristics in four groups**. Based on available clinical data, the colonoscopy populations were stratified into three risk groups; average risk group included those who did not have present, past or family history of CRC, and whose colonoscopy outcome was normal. High risk group still did not have CRC but had a risk factor of developing CRC such as advanced polyps, inflammatory bowel disease, positive past or family history of CRC, or high hereditary risk of CRC. Intermediate group includes subjects discovered with one or two polyps of low grade or having a single 2nd degree relative with CRC. In addition, this table shows that the high risk group constituted the largest portion of colonoscopy population. Finally, the mean age of the three risk groups do not differ from each other while the mean age of cancer positive patients were significantly older than non-cancer groups.

Figure (9) compares the mean level of GTA-446 among the four groups, the intermediate and high risk population did not differ significantly from the average risk population ($p > 0.5$), the cancer positive group had a statistically lower mean level of GTA-446 (mean = 0.77 SSS eq) compared to the average risk population (mean = 1.33 SSS eq, $p < 0.01$).

The rationale for conducting the comparison test is the expectation of finding a decline in

the mean level of GTA-446 as risk for developing CRC increases which was not the case here.

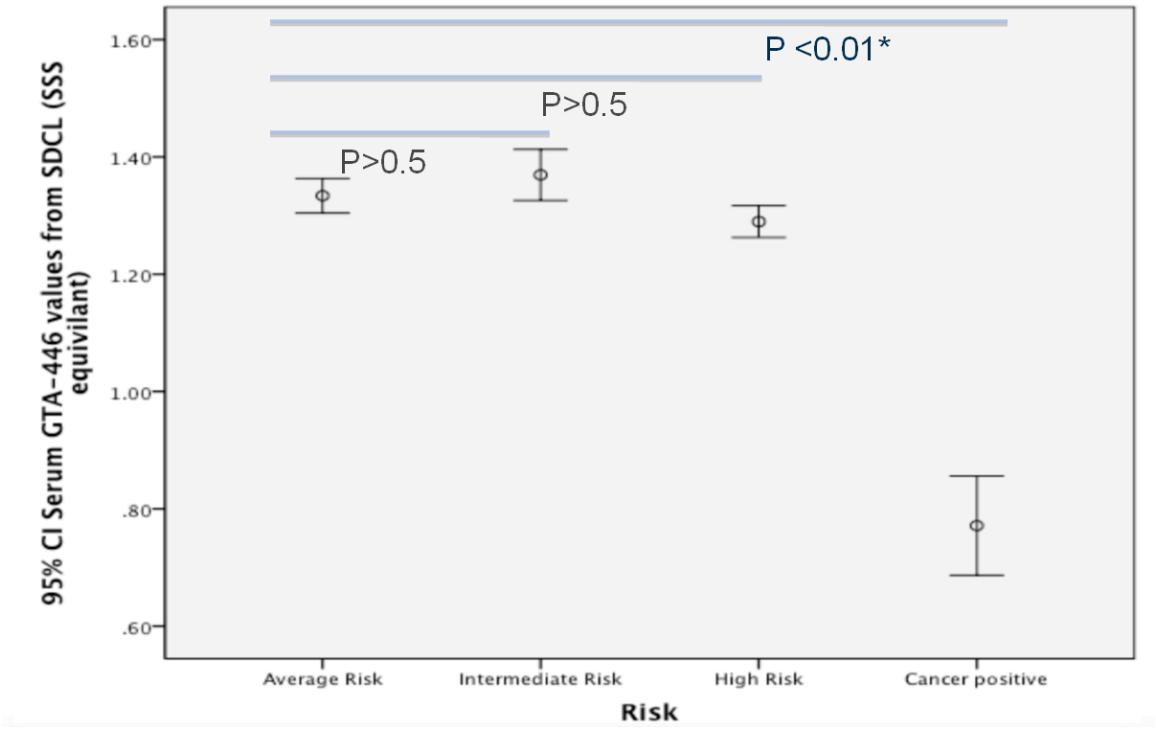


Figure (9) Distribution of the mean level of GTA-446 in all study groups with error bars representing 95% confidence interval. This figure compares the mean level of GTA-446 in intermediate risk (mean±SD=1.6 ±0.63 SSS eq), high risk (mean±SD =1.28 ±0.62 SSS eq.) and cancer positive groups (mean±SD = 0.77 ±0.41 SSS eq.) to average risk population (mean±SD = 1.33 ±0.62 SSS eq.). No significant differences were found among the three risk groups, cancer positive patients had a significantly lower level of GTA-446 than average risk group ($p<0.01$).

10.2 Correlation with age

The average risk colonoscopy population (N=1706) was further investigated for the relationship between age and GTA-446 biomarker (Table 4). A significant negative

correlation between age and serum GTA-446 biomarker level was found (r = -0.20) which confirms of what has been published before (297)). The same finding was reproduced examining the results from PDI (r = -0.19).

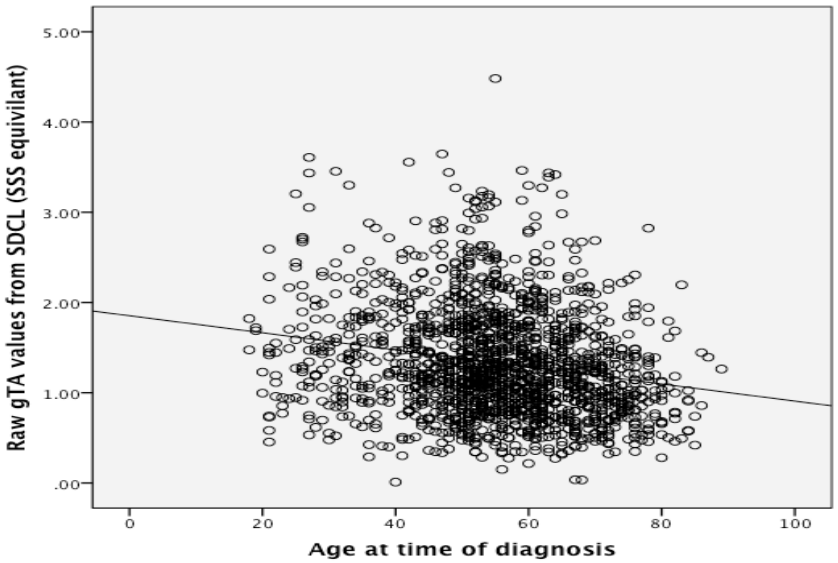


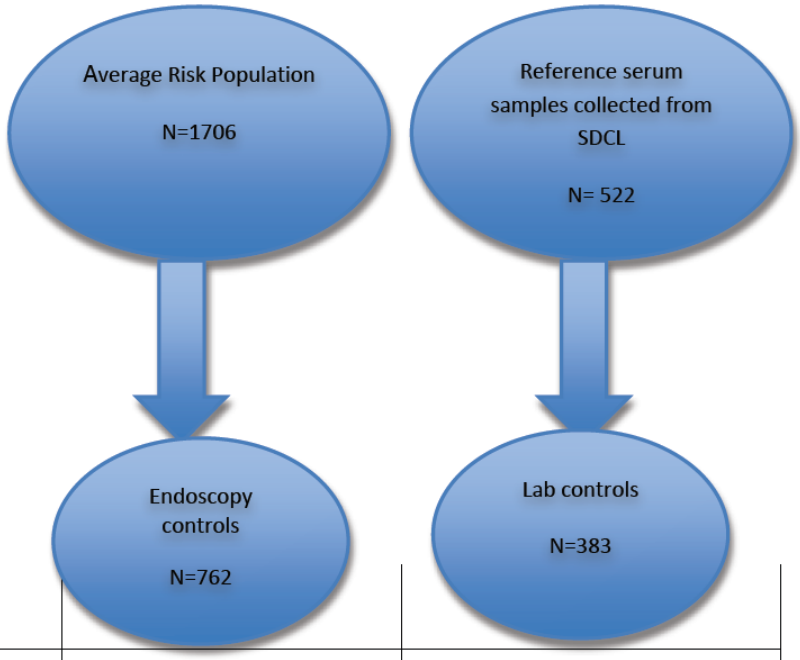
Figure (10) **A scatter dot diagram showing the correlation between age and individual serum levels of GTA-446 for SDCL average risk population.**

		Serum GTA-446 values from SDCL (SSS equivilant)	Serum GTA-446 values from PDI (ug/mL CAE eq)
Age at time of diagnosis	Pearson Correlation	-.20**	-.19**
	Sig. (2-tailed)	< 0.01	< 0.01

Table (4) **Correlation between age and serum level of GTA-446 for both SDCL and PDI data**. This table shows that there is a significant decline in the level of GTA-446 in the serum as age increases, the Pearson Correlation coefficient examining the data from both SDCL and PDI are very close (r= -0.2 and -0.19) respectively, confirming this relationship.

10.3 Selection of controls

Two sets of controls were used to calculate GTA-446 performance as a biomarker of colorectal cancer screening: Endoscopy controls and Lab controls. Endoscopy controls (N=762) were randomly sampled from the larger average risk population to be age matched to cancer positive subjects (N=94). Similarly, lab controls (N=384) were obtained from samples that were collected from SDCL along with age and gender data (N=522). Selection of controls was carried out using Fuzzy extension integrated into SPSS version 20 (Table 5). The rationale for choosing endoscopy controls is that they are considered superior to lab controls in terms of availability of clinical and pathological data and that they have gone through the same colonoscopy experience including the bowel preparation protocol as cancer-positive subjects.



Gender Males Females	 361 401	 115 268
Age (years) Mean/Range	 63.7 (28-89)	 63.4 (28-94)
Serum GTA level (SSS eq) (mean±SD)	 1.3 ± 0.6	 1.8 ± 0.8
T test	T statistic = 11.7 95% CI = 0.5 – 0.6, p < 0.01	

Table (5) **shows selection of endoscopy and lab controls with age, gender data and mean level of GTA-446 (SSS eq).** This table shows that the mean level of serum GTA-446 in endoscopy controls (mean =1.3 SSS eq) is significantly lower than that in lab controls (mean = 1.8 SSS eq, $p < 0.01$).

10.4 Establishment of cut-off levels for GTA-446 biomarker

An ROC curve was plotted to calculate area under the curve (AUC) to determine the best cut-off value that results in an acceptable sensitivity level above 85%. The AUC represents the probability that the GTA-446 result for a randomly chosen positive case will be lower than the result for a randomly chosen negative patient, an AUC equals 0.5 means that probability is not better than chance.

Comparing GTA-446 values for cancer positive patients against lab controls (Figure 11) results in an AUC of 0.90 (95% CI = 0.87 – 0.92. $p<0.01$), choosing 1.21 SSS equivalent as a cut-off results in sensitivity of 87.2% and specificity of 75.5% (Table 6).

Next, cancer positive subjects were compared against endoscopy controls to plot the ROC curve, this results in an AUC of 0.77 (95%CI = 0.74 – 0.80) and comparing it to the data from PDI, no significant difference was found between two AUCs (AUC from PDI = 0.77, 95%CI = 0.76 – 0.80, $p >0.5$)(Figure 12).

Using the same GTA-446 cut-off value used for lab controls (cut-off = 1.21 SSS equivalent) to calculate test performance characteristics comparing cancer positive subjects to endoscopy controls results in sensitivity of 87.2% and 86.2% for data analyzed by SDCL and PDI respectively. Specificity drops to 49.4% and 50.3% for SDCL and PDI respectively.

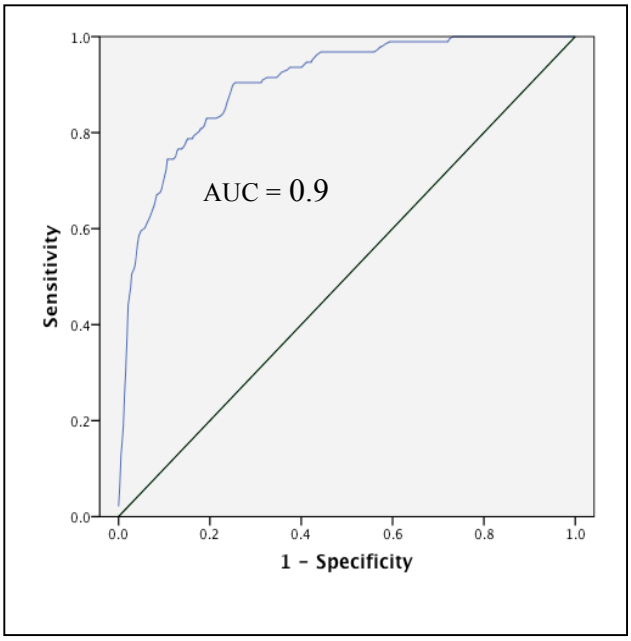


Figure (11) **Receiver Operating Characteristic (ROC) curve comparing GTA-446 levels in serum of cancer positive subjects (N=94) to lab controls (N=383)**. In this ROC curve, the true positive rate (sensitivity) is plotted as a function of the false positive rate (1—Specificity) for different cut-off points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. Also, the ROC curve is used to calculate the area under the curve (AUC), which represents the probability that a cancer positive case will have a GTA-446 level lower than a non-cancer case. The further the curved line is from the diagonal reference line, the greater the probability and the discriminatory power of the biomarker. In this figure, AUC of the biomarker equals 0.9 which is statistically significant ($p < 0.01$) (305).

AUC	Positive CRC if less than or equal to Cut-off (SSS equivalent)	Sensitivity		Specificity		Likelihood Ratio		Predictive Value	
		%	95% CI	%	95% CI	Pos	Neg	Pos %	Neg %
0.90	1.21	87.2	78.8 – 93.2	75.5	70.8 – 79.7	3.6	0.2	1	99

Table (6) **Summary of cut-off line, AUC with calculated test performance characteristics using lab controls as cancer-free subjects.** Predictive values were calculated based on the estimated CRC prevalence of 0.03% (306).

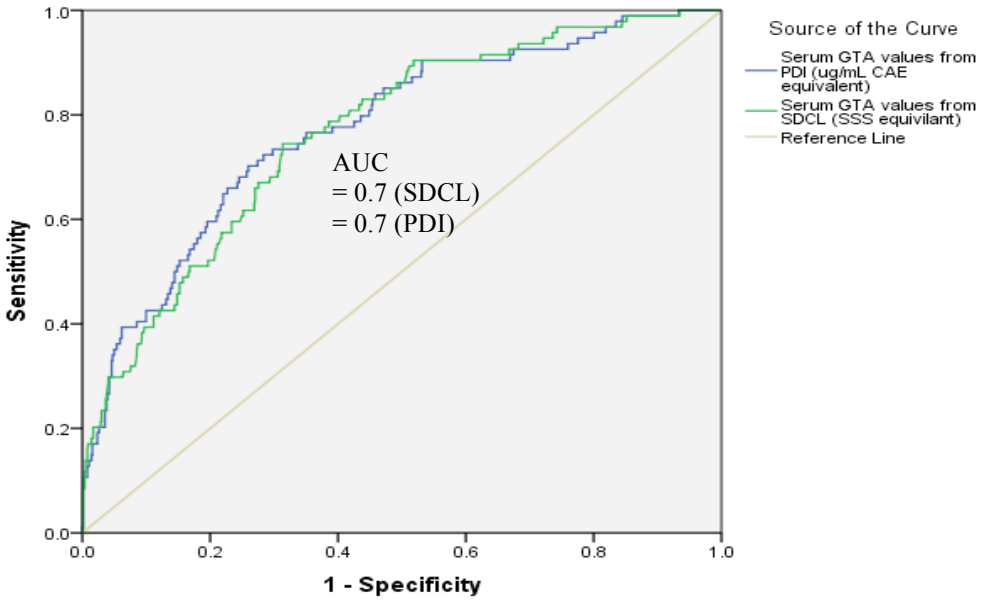


Figure (12) **Receiver Operating Characteristic (ROC) curve comparing GTA-446 levels in serum of cancer positive subjects (N=94) to Endoscopy controls (N=762) for SDCL and PDI data.** In this ROC curve, the true positive rate (sensitivity) is plotted as a function of the false positive rate (1—Specificity) for different cut-off points. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. Also, the ROC curve is used to calculate the area under the curve (AUC) which represents the probability that a cancer positive case will have a GTA-446 level lower than a non-cancer case. The further the curved line is from the diagonal reference line, the greater the probability and the discriminatory power of the biomarker. In this figure, AUC calculated using both SDCL and PDI data equals 0.7.

	AUC	Positive CRC if less than or equal to Cut-off	Sensitivity		Specificity		Likelihood Ratio		Predictive Value	
			%	95% CI	%	95% CI	Pos	Neg	Pos %	Neg %
SDCL	0.7	1.21 SSS equivalent	87.2	80 – 94	49.4	45.7 - 53	1.7	0.2	1	98
PDI	0.7	0.35 ug/mLCAE	86.2	77.5 – 92.4	50.3	46.7 – 53.9	1.7	0.3	1	98

Table (7) **Summary of cut-off line, AUC with calculated test performance characteristics using Endoscopy controls as cancer-free subjects.** Predictive values were calculated based on the estimated CRC prevalence of 0.03% (306).

Calculating the number of cases who fall below the cut-off value of 1.21 SSS eq., 87% of colorectal cancer patients were considered true positives compared to 50.5% and 24.5% for endoscopy and lab controls respectively who were considered false positives (Table 8 ,$p < 0.01$ after case weighting for both endoscopy and lab controls in comparison to cancer positive patients) .

Comparing the group of cancer positive patients to cancer-free endoscopy controls, the odds of having colorectal cancer when GTA-446 is positive equals 6.7 (95%CI = 3.6 to 12.4) and the odds ratio rises to 21 when comparing the same cancer positive patients to lab controls (95% CI = 10.9 to 40.2).

	Cancer positive patients (N=94)	Endoscopy controls (N=762)	Lab controls (N=383)
N of cases positive for GTA-446 (%)	82 (87.2%)	385 (50.5%)	94 (24.5%)
Pearson Chi-Square		X^2 (1,N=856) = 45.2 OR = 6.7, p<0.01	X^2 (1,N=477) = 127.4 OR = 21, p <0.01

N=number, OR=Odds Ratio

Table (8) **Number of cases below the cut-off (≤ 1.21 SSS eq) and considered positive for GTA -446**. This table demonstrates that 87.2% of true cancer-positive subjects were successfully detected by the GTA-446 biomarker while in lab and endoscopy controls, 24.5% and 50% of cases respectively were found to be false positives. Using Chi-square test to compare each of lab and endoscopy controls to cancer -positive subjects, (OR = 21 and 6.7 respectively) that is the odds of having cancer in a positive GTA-446 test sample is statistically significantly greater than not having colorectal cancer (p<0.01).

10.5 Comparison of SDCL and PDI's results

A strong correlation was found between GTA-446 values analyzed in SDCL versus those analyzed in PDI, (r = 0.82, p <0.01), and calculating true positive (TP), true negative (TN), false positive (FP) and false negative (FN) cases based on the established GTA-446 cut-off value (1.21 SSS eq for SDCL data and 0.35 ug/mL CAE for PDI's as determined by their researcher) results in a kappa statistic of 0.53 and p <0.01 which rejects the null hypothesis that there is no agreement between both results.

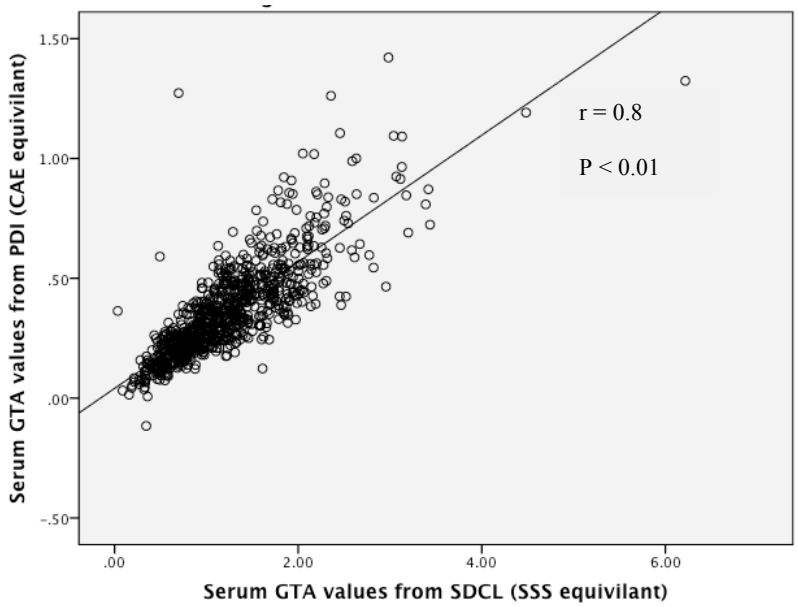


Figure (13) Scatter dot plot showing the relation between SDCL and PDI results. This figure shows a correlation coefficient of 0.8 between individual GTA-446 levels for SDCL (SSS eq.) on the horizontal axis and PDI's (ug/mL CAE) on the vertical axis, a statistically significant correlation was found ($p < 0.01$).

SDCL * PDI Crosstabulation

Count		SDCL				Total
		FN	FP	TN	TP	
PDI	FN	10	0	0	3	13
	FP	0	303	76	0	379
	TN	0	82	301	0	383
	TP	2	0	0	79	81
Total		12	385	377	82	856
Measure of Agreement	Kappa	0.53				
	P(sig.)	< 0.01				

TP = true positive, TN = true negative, FP = false positive, FN = false negative

Table (9) **Number counts of TN, TP, FP, FN for both SDCL and PDI and kappa measure of agreement** Using a cut-off of 1.21 SSS eq. for SDCL and 0.35 ug/mL CAE for PDI , the number of TP, TN, FP, and FN cases were counted and compared between SDCL and PDI's data. Kappa as a measure of agreement equals 0.53 with statistical significance ($p < 0.01$) which rejects the null hypothesis that there is no agreement between the two sets of results.

10.6 PDI's approach to data analysis

PDI's approach to data analysis of the study can be summarized as follow;

- They defined the cut-off value of GTA-446 as a serum concentration falling within the bottom 10^{th} percentile of reference samples (serum samples collected from SDCL along with age and gender data) with low age-associated risk (those aged 40-49, 0.35 ug/mL CAE).
- Based on the established 0.35 ug/mL CAE cut-off value, 86% of CRC positive cases were calculated as true positives, they also determined the relative risk, based on the proportions of CRC and control cases with low versus normal GTA-446 levels by decade of life for the reference and total colonoscopy populations with the assumption that the CRC incidence in the reference population would have been negligible.
- Finally, they compared the CRC incidence rate among subjects undergoing colonoscopy with low versus normal GTA-446 levels by decade of life.

It is concluded that PDI considered the current study a follow up one, and calculated relative risk and incidence rate to explain their results. Meanwhile, in the current thesis the new biomarker was validated as a diagnostic test and a screening tool for colorectal cancer that has been tested in a wide-scale case-control study. As a result, parameters such as sensitivity, specificity and odds ratio were calculated which are consistent with the concept of a case-control study. Both approaches are ways to analyze the data from two different perspectives that do not contradict each other.

11 DISCUSSION

Colorectal cancer is the second leading cause of cancer death in Canada with an estimation of 22,200 people having developed CRC in 2011 and 8.900 who died of the disease (307).

Early detection of CRC at early stages significantly improves patient outcome. While five-year survival rates reach 90% or higher for localized cancer, survival rates drop to 68% for regional cancer and 10% or less for metastatic cancer (308).

In Canada, the five-year relative survival ratio has improved considerably, rising by 7.7 percentage points to reach 63% in 2006. This may be attributed to higher prevalence of screening in the population (307).

Currently, the gold standard for CRC screening is colonoscopy due to its high sensitivity and specificity. However, colonoscopy holds several disadvantages with resulting poor patient compliance (309).

On the other hand, fecal screening tests lack the sensitivity and specificity of colonoscopy, and they require the patient to go through an unpleasant process of sample collection (310).

One strategy to improve patient adherence to CRC screening is to provide a test that is more convenient, minimally invasive, sensitive, and able to detect CRC at an early stage. Hence, there is a great need for new serum based biomarkers that fulfill the former criteria.

This study aims to validate GTA-446 as a serum based CRC screening test. Two sets of controls were used. The first; Lab controls (N=383) were serum samples collected from SDCL along with age and gender data, the second type of controls, Endoscopy controls (N=762) were obtained from the average risk population that were confirmed by colonoscopy to be pathology free and didn't have a higher risk of developing CRC than the average population.

It was found that the mean level of GTA-446 in Lab controls is significantly higher than Endoscopy controls. This could be attributed to the long bowel preparation that endoscopy control subjects had to go through before colonoscopy. Bowel preparation starts one day or two before the procedure by restricting solid diet and consuming a clear liquid diet which should not include food coloring or fatty substance (e.g. milk or creamer), preparation also involves administration of laxatives and/or enema to ensure complete bowel cleaning and subjects are instructed to start fasting after midnight the night before the test (311).

Another explanation for the difference in mean levels of GTA-446 between lab and endoscopy controls could be that although endoscopy control subjects were chosen to be free of colorectal, uterine, ovarian and breast cancer, other types of cancer were included which might have an effect on GTA-446 level, as the relationship between GTA-446 and other types of cancers have not been fully studied. Lastly, colonoscopy is the gold standard for CRC screening and detection, but a polyp miss rate has been reported especially if polyp size is small or polyp shape is flat (312).

The mean level of GTA-446 was not significantly different among average risk (N=1706, mean of GTA=1.33 SSS eq), intermediate risk (N=811, mean of GTA=1.36 SSS eq) or high risk population (N=2042, mean of GTA=1.28 SSS eq) but the mean level of GTA-446 was significantly lower in cancer positive subjects (N=94, mean of GTA=0.77 SSS eq) compared to the rest of the population groups which raises the possibility that the decline in GTA-446 is initiated by the cancer itself. This has not been investigated in the current study and more research is warranted.A key finding of the study is that serum GTA-446 was able to detect 87.2% of colorectal cancer cases at a specificity of 75.46% when compared to lab controls. Specificity drops to 49% when comparing colorectal cancer positive cases to endoscopy controls. This is expected given the significant difference in the mean level of GTA-446 among lab and endoscopy controls. In both cases, GTA-446 has a negative predictive value of over 96%.

This is in consensus with the performance of other serum biomarkers that have been tested mostly in case-control studies, some of these key markers include (313); colon cancer specific antigen with a sensitivity of 97.3% and a specificity of 78.4% (314), hypermethylated gene SEPT9 (which detects multiple methyl groups attached to cytosine residues in the DNA) was evaluated in two case-control studies and had sensitivities of 68-70% and specificities of 89-90% (315;316), and two markers for mRNA transcripts isolated from cells circulating in the blood (Guanylate Cyclase 2 C and Transmembrane 4 Superfamily member 1) had sensitivities of 74% and 78.6% and specificities of 95.2% and 100% respectively (317;318).

All these biomarkers have been tested in case-control studies with small sample size and have not been evaluated in a large population screening based study except for

biomarkers such as SEPT9 hypermethylation in the PRESEPT study, which evaluated the biomarker in a cohort study recruiting 7914 average risk subjects eligible for CRC screening by colonoscopy. Preliminary data from this study showed a combined sensitivity for three labs of 50% at 91% specificity (319).

The current SDCL-PDI study has several strengths.. First, is the large sample size of the study (N=4924); these subjects were tested for GTA-446 and their results were compared against colonoscopy results which is the gold standard for CRC screening and detection. Second, all serum samples have been extracted and analyzed independently twice at two distant facilities, and the results showed good correlation of GTA-446 values ($r=0.8$, $p < 0.01$), which adds to the reproducibility of the test method. Finally, all the samples were blindly extracted and analyzed at SDCL. The corresponding clinical results were only released after completion of the analysis and release of the results, which eliminates the possibility of observer's bias.

On the other hand, the study did not address the relationship between GTA-446 and other types of cancer or other medical conditions such as DM or the effect of fasting on the level of these biomarkers in blood. Given the low specificity of 49% of the biomarker when compared to endoscopy controls, a study looking into these associations is necessary.

Also, the average risk population was selected by controlling the unmodifiable risk factors such as; age, presence of polyps, family history, genetic conditions(e.g. familial adenomatous polyposis and lynch syndrome) and inflammatory bowel disease (e.g. ulcerative colitis and Crohn's disease). However, no data was collected regarding the modifiable risk factors to CRC such as diabetes, diet high in red meat, alcohol

consumption, obesity, smoking and level of physical activity. Lack of these data in an average risk population from whom endoscopy controls were selected should not be considered as a serious violation in study design as in real life situations an ideal biomarker should be able to detect cancer and not be impacted by other medical conditions.

12 CONCLUSION

Serum GTA-446 is a potential biomarker for minimally invasive detection of colorectal cancer that compares favorably to other serum based biomarkers. More research needs to be conducted to elucidate the relationship between GTA-446 and other medical conditions.

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