THE ROLE OF DIETARY EXPOSURE TO HETEROCYCLIC AROMATIC AMINES AND GENETIC SUSCEPTIBILITY IN COLORECTAL ADENOMA ETIOLOGY

By

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A thesis submitted to the Department of Public Health Sciences

in conformity with the requirements for the

Degree of Doctor of Philosophy

Queen's University

Kingston, Ontario, Canada

April, 2014

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Abstract

Background: Meat consumption is associated with an elevated risk of colorectal cancer (CRC); exposure to heterocyclic aromatic amines (HAAs), carcinogens produced when meat is cooked at high temperatures, is one hypothesized explanation for this relationship. HAAs form adducts with DNA; left unrepaired, DNA adducts can induce mutations which may initiate and/or promote the development of colorectal adenomas, precursors to the vast majority of CRCs. Along this continuum, genetic differences in the ability to biotransform or metabolize HAAs and repair DNA is postulated to modify the HAA-CRC relationship.

Methods: This thesis examined the HAA-CRC relationship in two studies (Phase 1 and 2). In a cross-sectional study of 99 healthy volunteers, Phase 1 investigated the relationship between dietary exposure to HAAs and the levels of bulky DNA adducts in blood leukocytes. In Phase 2, a cross-sectional study examined the relationships between dietary exposures to: a) HAAs and; b) meat mutagenicity, and the prevalence of colorectal adenomas among 342 patients undergoing a screening colonoscopy. Both Phase 1 and 2 examined potential gene-diet interactions between dietary HAAs and genetic factors relevant to the biotransformation of HAAs and DNA repair.

Results: In Phase 1, an interaction was observed for dietary HAAs and NAT1 polymorphisms where a positive association between HAA intakes and bulky DNA adduct levels was found among those with the NAT1 slow acetylator genotype, hypothesized to confer a lower ability to biotransform HAAs. In Phase 2, polymorphisms in genes involved in the biotransformation of HAAs (CYP1B1 rs10012 and rs1056827) and DNA repair (XPC rs2228001) were found to determine colorectal adenoma risk. As well, gene-diet interactions were observed for dietary

HAAs/meat mutagenicity exposures and polymorphisms in CYP1B1 and XPD (rs13181 and rs1799793). Overall, a higher risk of colorectal adenoma was observed with higher HAA and/or meat mutagenicity exposures among those with polymorphisms which confer a greater activity to biotransform HAAs and/or a lower ability to repair DNA.

Conclusion: This research supports the contribution of dietary HAAs and genetic susceptibility to the risk of developing colorectal adenomas and highlighted bulky DNA adduct formation as a potential biologic pathway through which HAAs may influence cancer risk.

Acknowledgements

People always say that acknowledgements and thank you's should be short and sweet. It is hard to stick to such a notion since throughout this journey I was supported, encouraged and inspired by so many people – and as I write I feel how words fall short on expressing gratitude on this scale of enormity.

I first and foremost must acknowledge my supervisors Will King and Tom Massey. Will, it is hard to sort out how much of who I am as a researcher was inspired by you. You have a humble approach to mentoring that made me chase after every idea and inspiration, and your support has been felt every step of the way. Your love of science is infectious and I thank you for always challenging me to see the "big" picture and insisting that the path less taken is always worth it in the end. It is hard to find one's calling in life – especially one so fitting and challenging, and I not only had one great supervisor to guide me, but two. Tom, you are ever-wise and knowing; you set the best example of balance. Your students' respect of you (including mine) is so apparent and you deserve every bit of it.

The Department of Public Health Sciences at Queen's houses the best faculty in the world and has been a second home to me. Harriet Richardson started it all by writing my letter of recommendation for Grad Studies at Queen's – thank you Harriet, for always going above and beyond in supporting me through my graduate journey. To my cherished professors, Kristan Aronson, Duncan Hunter, Patti Groome and Will Pickett: a big thank you for shaping my mind and always forcing me to take a leap.

I have met some of the greatest people and dearest friends here at my time at Queen's. Janet Ashbury: you are forever my Epidemiology mom; Sarah Peacock: my partner in crime and my rock; and Celine Simmers: my bestest friend who always believe in me more than I deserve.

Alyson Mahar: you kept me smiling and in love with science; Marianne Gee and Anne Grundy: I could never have asked for better study buddies; and all the rest of the gang, you kept me sane and relentless.

For my PhD, I was fortunate to be able to work on (what I hope) is the beginning of my life's work. This work was made possible by funding support from Cancer Care Ontario and the Canadian Cancer Society. As well, I must acknowledge the valued participants of both my projects without which this research would not have been possible.

Finally in this "short" acknowledgement, to my parents whose only requirement for me was to find happiness in life – thank you for the impossible task that keeps evolving day by day but of which I never lose sight of. To my sisters, as a middle child it is easy to thank the older one for the wisdom and the younger one for keeping me cool. And, I must acknowledge my husband Fred: you know all my faults and darkest doubts but still believe in me every day. You let me chance my dreams and paths that don't always work out so well but in the end you were always there and for that you get half a PhD.

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List of Abbreviations

ADPRT	ADP-ribosyltransferase
APE1	Apurinic/apyrimidinic endonuclease 1
ATM	Ataxia telangiectasia mutated
BER	Base excision repair
BMI	Body mass index
C	Cytosine
CCS	Canadian Cancer Society
CHARRED	Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease mutagen database
CI	Confidence interval
CRC	Colorectal cancer
CYP	Cytochrome P450
DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
ERCC1	Excision repair cross-complementing group 1
ERCC6	Excision repair cross-complementing group 6
FFQ	Food frequency questionnaire
GST	glutathione S-transferase
HAA	Heterocyclic aromatic amines
HDL	High density lipoprotein
HR	Homologous repair
IBS	Irritable bowel syndrome
ID	Identification
Log	Logarithmic
MAF	Minor allele frequency
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
MET	Metabolic equivalent of tasks
MS	Mass spectrometry
NAT	*N*-acetyltransferase
NBS1	Nijmegen breakage syndrome protein 1
NER	Nucleotide excision repair
OR	Odds ratio
PAH	Polycyclic aromatic hydrocarbons
PAR	Population attributable fraction
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
P53	Tumour protein 53
PR	Prevalence ratio
Q-Q	Quantile-quantile
RR	Relative risk
SD	Standard deviation

SNP	Single nucleotide polymorphism
SULT	Sulfotransferase
T	Thymine
UGT	UDP-glucuronsyl transferase
XPA	Xeroderma pigmentosum group A
XPC	Xeroderma pigmentosum group C
XPD	Xeroderma pigmentosum group D
XRCC1	X-ray repair cross-complementing group 1
XRCC3	X-ray repair cross-complementing group 3

Chapter 1: General Introduction

1.1 Summary of Research

This research investigated the contribution of dietary and genetic determinants to cancer risk using molecular epidemiologic approaches. Specifically, the role of heterocyclic aromatic amines (HAAs), a class of meat-derived carcinogens, and genetic factors that may contribute to the susceptibility to HAAs on colorectal cancer (CRC) risk was investigated.

Figure 1 outlines the biologic hypothesis of the role of HAAs in colorectal carcinogenesis. The underlying carcinogenic process related to this research involves dietary exposure to HAAs through meat consumption, adduction of reactive HAA metabolites to DNA, DNA alterations such as mutations, the development of colorectal adenoma and finally CRC onset. Individual genetic susceptibility is postulated to play a role along the HAA-CRC continuum. Specifically, genetic variation conferring differences in the ability to biotransform or metabolize HAAs and repair DNA damage have the potential to modify the HAA-CRC relationship.

The ultimate goal of this research was to characterize a sequence of events within the relationship between HAAs and CRC using biomarkers as tools to elucidate a mechanism through which HAAs may affect carcinogenesis and to examine potential gene-environment interactions. This research contributes to a better understanding of the role of HAAs as causative agents potentially underlying the link between meat consumption and CRC risk.

This doctoral dissertation examined the HAA-CRC association in two phases (Phase 1 and 2). In a cross-sectional study, Phase 1 investigated the relationship between dietary exposure to HAAs and the formation of potentially carcinogenic bulky DNA adducts in blood. In Phase 2, a cross-sectional study investigated the relationship between dietary exposure to HAAs and the

prevalence of colorectal adenoma, precursor to the vast majority of CRCs. In addition, Phase 2 examined the effect of meat mutagenicity in relation to colorectal adenoma prevalence; an investigation of meat mutagenicity or the total mutagenic activity in cooked meats provides a summary measure of exposure to all dietary HAAs as well as other meat-derived carcinogens. Both Phase 1 and 2 examined potential gene-environment interactions between dietary HAAs and genetic factors in relation to bulky DNA adducts (outcome of interest for Phase 1) and colorectal adenoma risk (outcome of interest for Phase 2).

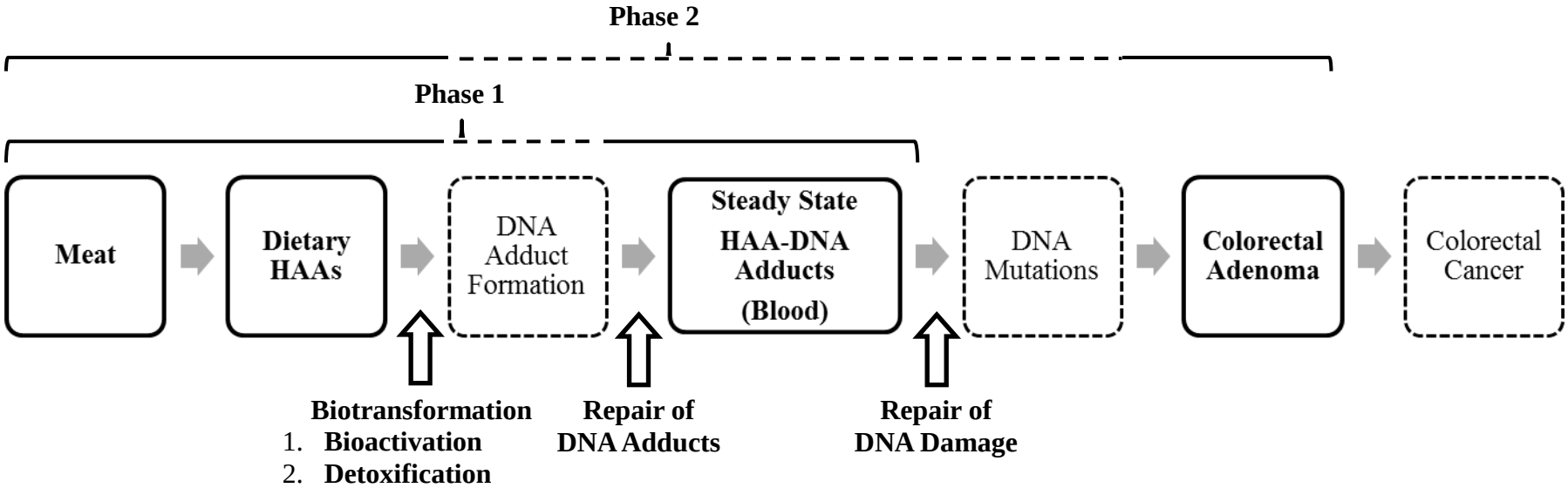

Figure 1.1: Conceptual model illustrating the goals of this doctoral research and the underlying biologic hypothesis of the role of HAAs in colorectal carcinogenesis. Solid lines indicate measured variables of interest in this dissertation.

1.2 Rationale

CRC is a multifactorial disease determined jointly by genetic susceptibility and exposure to environmental factors. The National Cancer Institute estimates that at least two thirds of CRCs are potentially preventable; as a result, there are tremendous opportunities for helping people avoid this cancer (1). Worldwide, CRC is the third most common cancer with over 1.2 million new cases occurring in 2008 (2). In Canada, CRC remains the third most common cancer and the second leading cause of death from cancer in both men and women (3).

Epidemiologic evidence has provided support for the association between meat consumption and CRC risk. Research in the fields of epidemiology, toxicology and population genetics has contributed to elucidating the hypothesized biologic relationship between meat consumption and the risk of CRC through a focus on meat-derived carcinogens like HAAs. HAAs are carcinogenic compounds formed when meat is cooked at high temperatures; generally, increases in CRC risk have been found among those who consume diets with high levels of HAAs and those who are genetically susceptible to the carcinogenic potential of HAAs. Studies have also shown that cooking practices largely determine dietary HAA exposure therefore exposure to HAAs is a modifiable risk factor that may have immense public health implications.

Though the biologic support for HAAs in colorectal carcinogenesis is strong, population-based studies have failed to report consistent and convincing magnitudes of effect to unequivocally support the relationship between HAA intake and CRC risk. Inconsistencies and weak associations may in part be attributable to inaccurate measurements of dietary HAAs, the long latency occurring between exposure to HAAs and cancer development, and variation in individual susceptibilities to HAA exposure. This doctoral dissertation combined epidemiology and molecular biology approaches to investigate the role of dietary HAAs in colorectal

carcinogenesis in two studies (Phase 1 and 2). The examination of the biologic relationship between dietary exposure to HAAs and DNA adduct formation, purpose of Phase 1, will inform on the biologic mechanism of HAAs in carcinogenesis. As well, the examination of the HAA/meat mutagenicity-colorectal adenoma relationship while considering the contribution of individual susceptibility, purpose of Phase 2, will further elucidate and clarify this relationship.

It is important to determine whether dietary factors like HAAs contribute to the etiology of CRC. The identification and understanding of modifiable practices associated with meat consumption would provide the basis for the development of CRC prevention initiatives that specifically target the reduction of HAA formation in common meat-cooking practices (4).

1.3 Objectives and Hypotheses

This doctoral dissertation examined the role of HAAs in colorectal carcinogenesis in two epidemiologic studies (Phase 1 and 2). Originally, the objectives in both Phases was to detect and measure specific HAA-DNA adducts as a biomarker of early biologic effect for Phase 1, and a biomarker of exposure for Phase 2. However, after extensive method development and validation, the quantification of specific HAA-DNA adducts was deemed to not be feasible.

Therefore, Phase 1 of this research, funded by Cancer Care Ontario, was revised to investigate the relationship between dietary exposure to HAAs and the levels of potentially carcinogenic bulky DNA adduct levels (a more global measure of adducts which incorporates HAA-DNA adducts) in blood leukocytes in a cross-sectional study of 125 healthy volunteers. In Phase 1, whether the relationship between dietary HAA intake and DNA adduct levels is modified by variation in genes involved in the biotransformation or metabolism of HAAs, and the repair of DNA adducts was also evaluated. An understanding of the relationship between

dietary exposure to HAA levels and DNA adducts in blood leukocytes contributes to the assessment of the potential role of HAAs in carcinogenesis.

Originally, the objective of Phase 2 of this doctoral dissertation, funded by the Canadian Cancer Society, was to investigate the relationship between specific HAA-DNA adduct levels measured in blood and normal-appearing colorectal muscosa, and colorectal adenoma risk. Due to the inability to quantify specific HAA-DNA adducts, the objective of Phase 2 was then revised to examine, in a cross-sectional study of 342 patients undergoing a screening colonoscopy, the relationships between: a) exposure to dietary HAAs and colorectal adenoma prevalence and; b) exposure to meat mutagenicity on the risk of colorectal adenomas. Specifically, meat mutagenicity was examined to facilitate an investigation of the effect of all dietary HAAs, in addition to other meat-derived carcinogens, in relation to colorectal adenoma prevalence. Phase 2 also examined the role of polymorphisms in genes involved in the metabolism of HAAs and DNA repair in modifying the HAAs/meat mutagenicity-colorectal adenoma relationship. The purpose of Phase 2 was to determine whether HAAs play a role in colorectal carcinogenesis through an influence on the risk of developing colorectal adenomas and whether this association is modified by variation in genetic susceptibility.

i. Phase 1: Dietary HAA Intake and Bulky DNA Adducts

Objective #1 determined the relationship between dietary HAA intake and DNA adduct levels measured in blood leukocytes. It was hypothesized that a strong relationship would exist between dietary HAAs and DNA adducts in blood; as well, DNA adduct levels were hypothesized to be a function of genetic susceptibility and other lifestyle, demographic and dietary factors.

Objective #2 investigated whether the relationship between dietary HAAs and DNA adducts in blood leukocytes were modified by variation in activity to biotransform HAAs and repair DNA adducts. It was hypothesized that the relationship between dietary HAAs and DNA adducts would differ according to variation in genes that convey a different activity to biotransform HAAs and/or repair DNA adducts.

ii. Phase 2: Dietary HAA Intake and the Risk of Colorectal Adenoma

Objective #1 investigated whether a relationship existed between: a) dietary exposure to HAAs and the prevalence of colorectal adenomas and; b) meat mutagenicity exposure and colorectal adenoma prevalence. It was hypothesized that those with higher exposure to HAAs or meat mutagenicity would have a higher risk of colorectal adenoma.

Objective #2 determined whether the relationships between: a) dietary exposure to HAAs and the prevalence of colorectal adenomas and; b) meat mutagenicity exposure and colorectal adenoma prevalence (Objective #1) were modified by variation in genes involved in the biotransformation of HAAs and DNA repair. It was hypothesized that the relationships between dietary HAAs/meat mutagenicity and the prevalence of colorectal adenoma would differ according to variation in genes that convey a different activity to biotransform HAAs and/or repair DNA.

1.4 Context of Research

This doctoral dissertation was conducted within the framework of an interdisciplinary cancer research team with combined expertise in molecular epidemiology, mechanistic toxicology and genetics. The student investigator (V. Ho) was responsible for the conceptualization of the research questions, and the design and conduct of the study methods under the mentorship of Drs. W. King and T.E. Massey. She has been highly involved in writing

all research proposals submitted for funding Phase 1 and 2, and is a co-investigator on both operating grants obtained. For Phase 1, the student investigator developed the study instruments and laboratory protocols. She was jointly responsible for the recruitment of 125 healthy participants and DNA isolation along with PhD candidate (S. Peacock) who had a separate focus of utilizing Phase 1 subjects to investigate the relationship between telomere length and bulky DNA adduct levels in blood leukocytes. For Phase 2, the student investigator contributed to the development of the study instrument and was involved in the development of the protocols for study recruitment. For both Phase 1 and 2, the student investigator developed separate gene-selection strategies and coordinated genotyping of all polymorphisms of interest; she was also responsible for the development and conduct of complex analysis strategies that merged molecular and genetic information with epidemiologic methods.

1.5 Thesis Outline

This thesis is written in a traditional thesis format and is organized in 8 sections. A review of the relevant literature for the HAA-CRC relationship is presented in Chapter 2. Chapter 3 provides a description of the gene-selection strategies used to identify genetic factors of interest for Phase 1 and 2. An overview of methods including study design, development of laboratory protocol and statistical analysis strategies are presented separately for Phase 1 (Chapter 4) and Phase 2 (Chapter 6). Chapter 5 and Chapter 7 present the results of the data analysis for Phase 1 and 2, respectively. Finally, a discussion of results, methodological limitations and future directions are presented in Chapter 8.

1.6 References

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Chapter 2: Literature Review

Heterocyclic Aromatic Amine-Colorectal Cancer Relationship

Evidence suggests that diet plays a major role in the etiology of colorectal cancer (CRC) (1). Studies examining the effect of high meat consumption on CRC have produced some inconsistent results, but the weight of evidence points towards an elevated risk. During the cooking of meats at high temperatures, heterocyclic aromatic amines (HAAs) are formed, and exposure to these carcinogens is one hypothesized explanation for the meat-cancer relationship. This Chapter presents the literature review on the HAA-CRC relationship.

2.1 Colorectal Adenoma – A Surrogate Endpoint for Colorectal Cancer

Experimental and observational studies support the use of colorectal adenomas as surrogate end points for CRC; it has been estimated that most cases (70-90%) of CRC are preceded by colorectal adenomas (2). Throughout this Chapter, which provides the literature review on the HAA-CRC relationship, investigations of colorectal adenoma etiology were considered interchangeably with those of CRC. A discussion on the use of colorectal adenoma as a surrogate end point for CRC is presented in Section 2.4.3.

2.2 Meat Consumption and Colorectal Cancer Risk

A recent review by the World Cancer Research Fund reported a summary relative risk (RR) of 1.16 (95% confidence interval (CI): 1.04-1.30) for CRC associated with each 100 g/day increase in red and processed meat intake (3). Red and processed meat consumption is associated with a number of factors that may increase cancer risk, including increased exposure to saturated fat and nitrosamines, and carcinogens that are formed during the cooking of meats such as HAAs and polycyclic aromatic hydrocarbons (PAHs) (4). In addition, a diet high in overall meat intake could result in limited consumption of fiber and other nutrients that may have

a protective effect on CRC risk (4). Thus, an understanding of the meat-CRC relationship requires isolation of potential causal factors such as HAAs.

2.3 Exposure Assessment Framework for Heterocyclic Aromatic Amines

Dose of an environmental exposure is often conceptualized as a continuum starting with available or external dose, to administered dose, to absorbed dose, to biologically effective dose and finally, to biologic and health effects (5). Figure 2.1 expands the conceptual model of this research into a more a detailed continuum of events linking dietary HAA exposures to CRC development. HAAs are formed during the cooking of meat by condensation of creatine with amino acids and sugars (6). Dietary exposure to HAAs is considered to be a potential cancer risk factor because these genotoxic compounds are produced in relatively high concentrations under normal cooking conditions and can form adducts with DNA that can induce DNA damage and enhance the process of carcinogenesis (6). The underlying carcinogenic process related to this research involves dietary exposure to meat and HAAs (available and administered dose), internalization of HAAs in the body (absorbed dose), adduction of reactive HAA metabolites to DNA (biologically effective dose), and biologic and health effects including DNA mutations, colorectal adenoma and CRC (7).

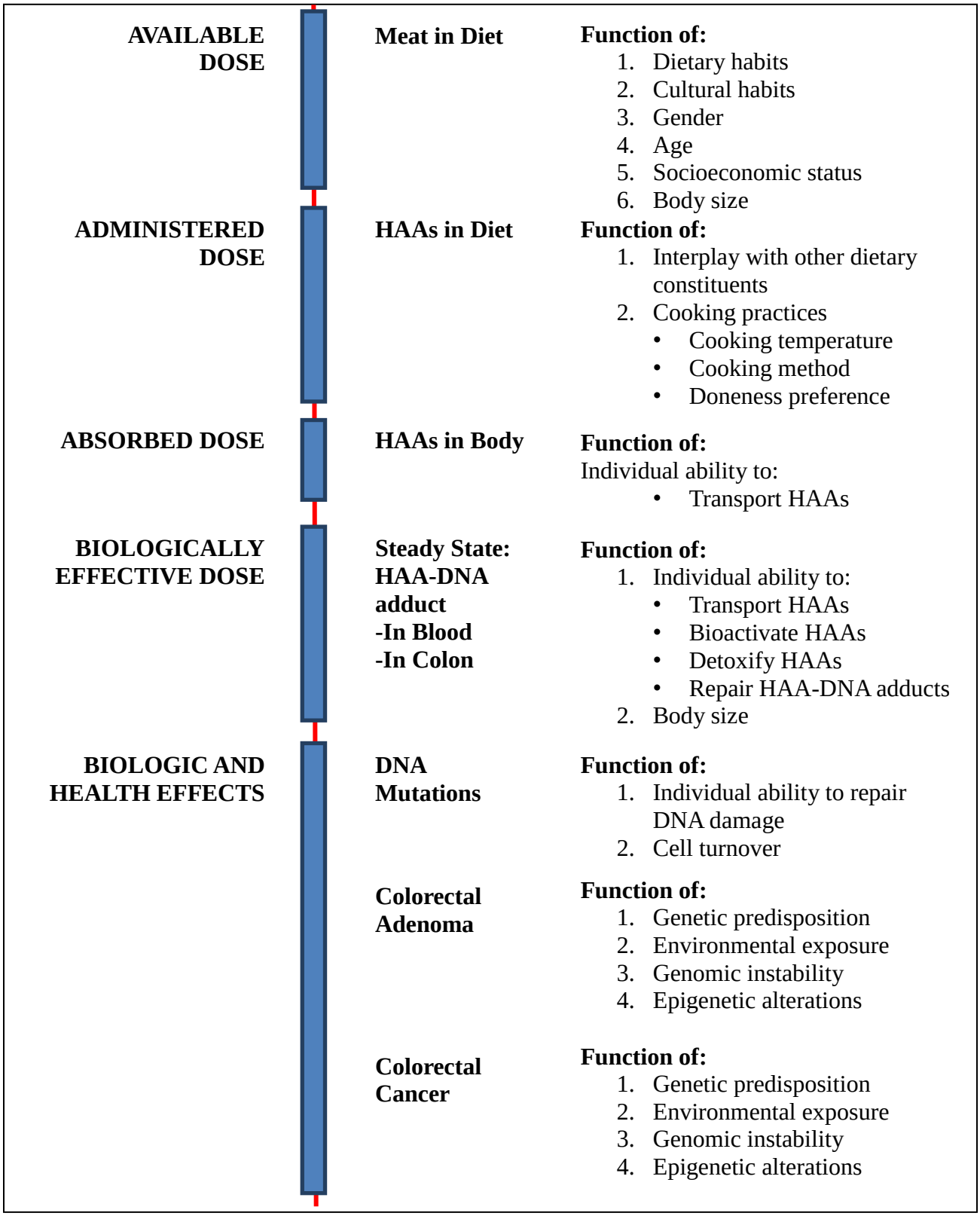

Figure 2.1: Underlying framework for HAA-CRC relationship

2.4 Colorectal Cancer

2.4.1 Descriptive Epidemiology

Worldwide, CRC is the third most common cancer with over 1.2 million new cases occurring in 2008 (8). Rates of CRC vary by country; a greater than three-fold difference in age-standardized CRC incidence rates is seen with higher rates observed in developed versus less developed countries (9). Evidence from migrant studies support that the geographic variation of CRC incidence rates could be attributable to lifestyle and environmental factors since individuals who move from countries with low rates to high rates of CRC show increased risk similar or close to those of the host country (9). In Canada, CRC is the third most common cancer and the second leading cause of death from cancer in both men and women with an estimated 23,900 new cases and 9,200 deaths for both sexes occurring in 2013 (10)

2.4.2 Risk Factors for Colorectal Cancer

It has been estimated that genetic syndromes such as familial adenomatous polyposis and hereditary non-polyposis colorectal cancer explain less than 15% of all CRC (9). Dietary habits have been estimated to account for 50% to 60% of all CRC (11). Factors such as high consumption of red and processed meat, caloric intake and alcohol are risk factors for CRC; in contrast, protective dietary factors for CRC include high consumption of dairy products, fiber, fruits and vegetables, and a high intake of calcium and vitamin D (9,12-16). Other non-dietary risk factors for CRC include male sex, cigarette smoking, exposure to environmental tobacco smoke, physical inactivity, previous diagnosis of inflammatory bowel disease, and a body mass index (BMI) of more than 25 kg/m^2 (9,14,17). As well, emerging evidence provides some support for the role of caffeine intake and biomarker levels of albumin, folate, lipid ratio, calcium and triglycerides (16,18-23). Finally, a reduced risk of CRC has been reported among

regular users of aspirin or other nonsteroidal anti-inflammatory drugs and hormone replacement therapy (9).

2.4.3 The Colorectal Adenoma-Carcinoma Sequence

Since the original hypothesis put forth by Hill and colleagues in 1975, evidence from the fields of epidemiology, physiology, cellular and molecular biology have accumulated to support the colorectal adenoma-carcinoma sequence, a widely accepted description focused on the transition of macroscopically normal mucosa through adenomatous polyp formation (adenoma) to cancer. Colorectal adenomas are benign precursor lesions that are visible on the mucosal surface of the colon and are characterized by dysplastic epithelium that "unequivocally represents neoplastic epithelial proliferation (24)". The following lines of evidence support the occurrence of the adenoma-carcinoma sequence (24).

First, temporal evidence is provided by the familial adenomatous polyposis syndrome, an inherited condition characterized by the development of hundreds to thousands of adenomas in the colon during adolescence or young adulthood; patients with familial adenomatous polyposis syndrome who are not treated by prophylactic colectomy develop CRC at a mean age approximately three decades younger than the general population (24). Second, histopathologic examination of adenomas sometimes has shown small areas of intra-mucosal adenocarcinoma within the larger benign lesion; thus, on a morphological level, adenomas appear to be the precursor lesion (24). Third, the distribution of adenomas in the colon parallels the distribution of CRC and epidemiologic studies have demonstrated increased rates of CRC among patients with adenomas as compared to the general population (24). Finally, prior to our present convention of removing colorectal adenomas upon detection during colonoscopy, detected polyps in the past were sometimes left untreated. In one study, the cumulative incidence of CRC

during follow-up of patients with adenomas that were not removed upon detection was 35% at 20 years (24).

Owing to the strong evidence supporting the colorectal adenoma-carcinoma sequence, increasingly, adenomas are being used as surrogate end points for CRC in epidemiologic research (25). A number of advantages exist in the use of colorectal adenomas as a surrogate end point including a shorter latency between exposure and event, a more common event and an elucidation of whether risk factors for CRC also relate to adenoma development. However, it is important to note some of the limitations of the colorectal adenoma-carcinoma model to better appreciate and weigh the implications of using colorectal adenoma as a surrogate end point for CRC (26). To be clear, the controversy surrounding the colorectal adenoma-carcinoma sequence does not challenge the fact that colorectal adenomas may progress to cancer; rather the discussion surrounds the proportion of CRCs that develop within a pre-existing adenoma (26). An individual adenoma has an extremely low probability of evolving into a CRC within an average human life span and evidence is increasing to support alternative pathways to CRC that implicates lesions other than that of traditional adenomas (26). Therefore, results of this investigation of the role of HAAs in CRC etiology using colorectal adenoma as a surrogate outcome should thus be interpreted with a caveat; specifically, findings from this research are expected to contribute to the understanding of CRC etiology but are likely limited to the proportion of CRCs that arise from existing adenomas.

2.5 Heterocyclic Aromatic Amines

2.5.1 Meat Consumption and Colorectal Cancer

The association between meat consumption and CRC has been investigated by numerous epidemiologic studies. Overall, studies have reported significant reductions in cancer risk among

those who avoided meat and increases in cancer risk among daily meat eaters (4,27). Due to the methodologic limitations associated with recall bias and the latency occurring between meat consumption and CRC development, studies that employed a prospective assessment of diet prior to disease diagnosis likely provide the best level of evidence for evaluating the meat-CRC association. Since 2000, five meta-analyses have assessed the association between meat consumption (including all meat or red meat or processed meat intake) and CRC risk evaluated in prospective cohort studies (28-33). The results of these meta-analyses support the hypothesis that high consumption of all meat, red meat and processed meat increases the risk of CRC (Table 2.1). It is important to note that the consideration of red versus processed meat intake may represent exposure to different carcinogens; specifically, the cooking of red meats at high temperatures produces high levels of HAAs and PAHs whereas processed meat contains N-nitroso compounds which have a distinct carcinogenic mechanism of action separate from HAAs and PAHs (34).

Table 2.1. Summary of five meta-analyses of prospective cohort studies investigating the risk of CRC associated with the intake of all meat, red meat and processed meat.

	All Meat	**Red Meat**	**Processed Meat**
Sandhu, 2001	RR=1.21 (1.10-1.33)[1]	RR=1.27 (1.13-1.46)[1]	RR=1.29 (1.20-1.49)[1]
Larsson, 2006	-	RR=1.28 (1.15-1.42)[2]	RR=1.20 (1.11-1.31)[2]
Alexander, 2010	-	-	RR=1.16 (1.10-1.23)[2]
Alexander, 2011	-	RR=1.12 (1.04-1.21)[2]	-
Chan, 2011		RR=1.22 (1.11-1.34)[2]	

[1]Relative risk associated with 100 g/day increase in meat intake
[2]Relative risk associated with highest vs. lowest meat intake category

2.5.2 Heterocyclic Aromatic Amines – A Potential Causal Factor Underlying the Meat-Colorectal Cancer Relationship

Over the past two decades of research investigating the meat-CRC association, the intake of meats prepared with high temperature cooking methods has emerged as a potential risk factor for CRC (35). One potential biologic hypothesis for this relationship is exposure to carcinogens

present in cooked meat. HAAs are one class of carcinogenic compounds that are formed when meat is cooked at high temperatures by the condensation reaction of creatine with amino acids and sugars (36).

Dietary exposure to HAAs is considered to be a potential risk factor for cancer due to the abundant production of these compounds even under normal cooking conditions and their mutagenic activity (36). In general, an increase in the quantity and frequency of meat consumption and the use of high-temperature cooking methods (pan-frying, oven-broiling and grilling/barbecuing) produce the highest HAA concentrations (36). In addition, the degree of doneness, which is often closely related to surface browning and total cooking time, are key issues for HAA production in cooked meats; specifically the optimal conditions for HAA formation include internal temperatures between 150ºC to 200ºC and greater external charring (36,37). To date, at least 20 HAAs have been identified in cooked foods; the three most abundant carcinogenic HAAs formed in meats are 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) (36,38). These three compounds have been detected in beef, lamb, pork, chicken and fish, especially when chargrilled, fried or roasted (36,38). PhIP and MeIQx have been demonstrated to be potent mutagens in bacterial and mammalian cell genotoxicity assays and have been shown to induce colon tumours in rats; less genotoxicity data is available for DiMeIQx (39-42).

2.5.3 Assessment of Heterocyclic Aromatic Amine Content and Meat Mutagenicity in Epidemiologic Research

Epidemiologic studies have utilized data on the frequency of meat consumption, preferred cooking methods and usual doneness levels as surrogate measures of HAA exposure. These are

important characteristics of meat intake because they in part determine HAA exposure and are modifiable practices. Recently, a more specific assessment of dietary exposure to HAAs has been made possible by the Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease mutagen database (CHARRED) developed by the National Institutes of Health (43). In the development of CHARRED, multiple samples of commonly-consumed meats were cooked by different methods and to varying degrees of doneness and analyzed for PhIP, MeIQx and DiMeIQx content as well as total mutagenic activity.

Meat mutagenicity or total mutagenic activity is a biological measure quantified using the Ames test (44); meat mutagenicity integrates the mutagenic potential of all the different classes of carcinogens, including HAAs, found in cooked meats. Meat mutagenicity as an exposure measure in epidemiologic research facilitates the investigation of the effect of <u>all or total</u> dietary HAAs acknowledging that other classes of carcinogens also contribute to meat mutagenicity. As discussed, dietary exposure to HAAs is considered to be a potential risk factor for cancer due to the abundance of these compounds found in cooked meats and their mutagenic potential (36). Therefore, the use of meat mutagenicity as a measure of exposure to total dietary HAAs in epidemiologic research is superior to the simple summation of PhIP, MeIQx and DiMeIQx concentrations (derived from the CHARRED mutagen database) since the sum of specific HAAs would be heavily weighted by the HAA that is most abundant in cooked meats and not account for the different mutagenic potentials of each HAA.

In the CHARRED mutagen database, data on specific HAA content and mutagenic activity for 120 categories representing the composite of different meat items, cooking methods and doneness levels are available (26). Utilizing the CHARRED mutagen database, levels of exposure to PhIP, MeIQx and DiMeIQx as well as meat mutagenicity can be assigned to

individuals on the basis of their self-reported frequency of meat intakes according to different cooking methods, preferred level of doneness and usual serving size.

2.5.4 Heterocyclic Aromatic Amines, Meat Mutagenicity and Colorectal Cancer – Epidemiologic Evidence

The intake of meats prepared with high temperature cooking methods has been used in epidemiologic research as a surrogate measure of HAA exposure and meat mutagenicity (35). Earlier studies typically only assessed the intake of a few items of high-temperature cooked meats in a food frequency questionnaire (FFQ) which provided an inadequate assessment of dietary exposure to HAAs and meat mutagenicity (35). More recent epidemiologic studies have attempted to additionally assess cooking methods and preference for doneness levels in estimating HAA exposure and meat mutagenicity (35). To standardize the assessment of doneness level some studies have used colour photographs for cooked meats that reflect a range of doneness levels from rare to very-well-done to supplement the FFQ (35). Finally, to estimate intake of specific HAAs and meat mutagenicity, recent epidemiologic studies have linked FFQ responses to the CHARRED mutagen database (45).

This literature review assessed the role of dietary exposure to HAAs and meat mutagenicity in colorectal carcinogenesis by considering both CRC and colorectal adenomas as outcomes of interest. Retrospective assessment comparing the exposure of dietary HAAs/meat mutagenicity among cases and controls has been the most common approach used to investigate the relationship between HAAs/meat mutagenicity and CRC/colorectal adenoma risk (27). To date, 12 case-control studies have been conducted in males and females to investigate the relationship between exposure to HAAs and the risk of CRC/colorectal adenoma (Table 2.2) (37,45-58); nine of which reported a statistically significant association. Among the 12 studies,

five also assessed the association between meat mutagenicity and the CRC/colorectal adenoma risk (37,53-55,58); four out of the five case-control studies report a statistically significant association. For HAAs, odds ratios (OR) comparing the highest to lowest HAA exposure category ranged from 0.6 to 3.1 for PhIP, 0.6 to 4.1 for MeIQx and 0.6 to 2.2 for DiMeIQx (37,46-58). For meat mutagenicity, ORs comparing the highest to lowest exposure category ranged from 1.3 to 3.1. The inconsistent results, particularly for HAAs, that have been reported may partly be due to methodological limitations including the potential for recall bias (37,46-58), low response rates (37,46,49,51,53,59,60), and the use of inaccurate instruments to capture exposures to HAAs and meat mutagenicity (37,46-57).

Six epidemiologic studies investigating the HAA-CRC/colorectal adenoma relationship through the assessment of dietary HAAs prior to a diagnosis of CRC/colorectal adenoma were identified (Table 2.3) (34,47,61-64); three report a statistically significant association. These studies are comprised of three nested case-control, two prospective cohort and one cross-sectional study design. Of the six studies, four studies also investigated the association between meat mutagenicity and CRC/colorectal adenoma (54,62,63,65), one of which reported a statistically significant association. Estimates of relative risk comparing the highest to lowest HAA exposure category ranged from 0.99-1.47 for PhIP, 1.09-1.90 for MeIQx and 1.17 to 1.21 for DiMeIQx. For meat mutagenicity, Cross et al., reported hazard ratios of 1.14 (1.01-1.20) for CRC when comparing the highest to lowest quintile of meat mutagenicity exposure. The assessment of exposure prior to diagnosis of CRC/colorectal adenoma is an obvious strength of these studies as it limits the potential for differential recall between events and non-events. However, the potential for misclassification of exposure exists as these studies did not collect information on serving size and/or doneness level using standardized instruments (34,47,61-64).

Table 2.2. Summary of results for case-control studies of HAAs exposure and CRC/colorectal adenoma risk

Author	Year	Study Outcome	Study Population	PhIP	MeIQx	DiMeIQx	Meat Mutagenicity
Augustsson	1999	Colon and rectal cancer	Colon: N_{Cases}=352 $N_{Controls}$=553 Rectal: N_{Cases}=273 $N_{Controls}$=249	Colon: OR=0.6 (0.4-0.9)[1] Rectal: OR=0.6 (0.4-1.1)[1]	Colon: OR=0.6 (0.4 -1.0)[1] Rectal: OR=0.7 (0.4-1.1)[1]	Colon: OR=0.6 (0.4-0.9)[1] Rectal: OR=0.6 (0.4-1.1)[1]	-
Sinha	2001	Colorectal adenoma	N_{Cases}=146 $N_{Controls}$=228	OR=2.5 (1.1-5.5)[1]	OR=2.1 (1.0-4.3)[1]	OR=2.2 (1.2-4.1)[1]	OR=3.1 (1.4-6.8)[1]
Le Marchand	2002	Colon and rectal cancer[2]	Colon: N_{Cases}=289 $N_{Controls}$=289 Rectal: N_{Cases}=137 $N_{Controls}$=137	Colon: OR=1.0 (0.6-1.6)[3] Rectal: OR=1.7 (0.3-3.8)[3]	Colon: OR=1.0 (0.6-1.1)[3] Rectal: OR=3.1 (1.3-7.7)[3]	Colon: OR=1.1 (0.7-1.7)[3] Rectal: OR=2.7 (1.1-6.3)[3]	-
Nowell	2002	Colorectal cancer	N_{Cases}=155 $N_{Controls}$=380	Data not shown	OR=4.09 (1.94-9.08)[4]	Data not shown	-
Butler	2003	Colon cancer	N_{Cases}=620 $N_{Controls}$=1038	OR=0.9 (0.6-1.5)[1]	OR=1.1 (0.6-2.0)[1]	OR=1.8 (1.1-3.1)[1]	OR=1.4 (1.0-2.0)[1]
Gunter	2005	Colorectal adenoma	N_{Cases}=261 $N_{Controls}$=304	OR=1.01 (0.58-1.73)[1]	OR=0.89 (0.52-1.55)[1]	OR=1.15 (0.69-1.91)[1]	-
Butler	2005	Colon cancer	N_{Cases}=400 $N_{Controls}$=412	OR=1.4 (1.0-1.9)[4]	OR=0.9 (0.6-1.4)[4]	OR=1.6 (1.1-2.3)[4]	-
Shin	2007	Colorectal adenoma	N_{Cases}=573 $N_{Controls}$=1544	Adenoma: OR=1.1 (0.8-1.4)[5]	Adenoma: OR=0.9 (0.7-1.2)[5]	Adenoma: OR=1.1 (0.8-1.4)[5]	Adenoma: OR=1.0 (0.8-1.4)[5]
Girard	2008	Colorectal cancer	N_{Cases}=537 $N_{Controls}$=866	OR=1.0 (0.7-1.3)[4]	OR=1.2 (0.9-1.7)[4]	OR=1.2 (0.9-1.7)[4]	-

Wang	2011	Colorectal adenoma and cancer	Colorectal adenoma: N_{Cases}=914 $N_{Controls}$=1185 Colorectal Cancer: N_{Cases}=496 $N_{Controls}$=607	Adenoma: OR=1.26 (0.98-1.62)[3] Cancer: OR=1.20 (0.86-1.68)[3]	Adenoma: OR=1.19 (0.92-1.54)[3] Cancer: OR=1.10 (0.78-1.54)[3]	Adenoma OR=1.37 (1.08-1.75)[3] Cancer: OR=0.99 (0.71-1.37)[3]	-
Fu	2011	Colorectal adenoma	N_{Cases}=1881 $N_{Controls}$=3764	OR=1.3 (1.1-1.5)[5]	OR=1.4 (1.2-1.7)[5]	OR=1.3 (1.1-1.6)[5]	OR=1.3 (1.1-1.6)[5]
Helmus	2013	Colorectal cancer	N_{Cases}=1062 $N_{Controls}$=1645	OR=1.38 (0.91-1.52)[5]	OR=1.87 (1.44-2.44)[5]	OR=1.68 (1.29-2.17)[5]	OR=1.77 (1.36-2.30)[5]

[1]Comparison of highest vs. lowest quintile
[2]Results are limited to men only; female results were not provided
[3]Comparison of highest vs. lowest tertile
[4] Comparison of above vs. below median
[5]Comparison of highest vs. lowest quartile

Table 2.3. Summary of results for studies (prospective cohort, nested case-control and cross-sectional studies) with assessment of HAAs exposure prior to CRC/colorectal adenoma diagnosis

Author	Year	Study Design	Study Outcome	Study Population	PhIP	MeIQx	DiMeIQx	Meat Mutagenicity
Sinha	2005	Nested case-control study	Colorectal adenoma	N_{Cases}=3696 $N_{Controls}$=34817	OR=1.11 (0.98-1.25)[1]	OR=1.08 (0.95-1.23)[1]	OR=1.05 (0.94-1.18)[1]	OR=1.08 (0.95-1.22)[1]
Wu	2006	Nested case-control study	Colorectal distal adenoma	N_{Cases}=581 $N_{Controls}$=13451 (Controls included those with proximal and/or rectal adenoma)	OR=1.11 (0.85-1.46)[1]	OR=1.28 (0.95-1.71)[1]	OR=1.08 (0.86-1.37)	OR=1.29 (0.97-1.72)[1]
Rohrmann	2009	Prospective cohort study	Colorectal adenoma	N_{Cases}=516 $N_{Controls}$=3699	RR=1.47 (1.13-1.93)[2]	RR=1.27 (0.97-1.68)[2]	RR=1.18 (0.92-1.53)	-
Nothlings	2009	Nested case-control study	Colorectal cancer	N_{Cases}=1009 $N_{Controls}$=1522	OR=1.03 (0.77-1.39)[3]	OR=1.09 (0.81-1.47)[3]	OR=1.18 (0.88.1.59)[3]	-
Ferrucci	2009	Cross-sectional study	Colorectal adenoma[4]	N_{Cases}=158 $N_{Controls}$=649	OR = 1.49 (0.85-2.62)[2]	OR = 1.90 (1.05-3.42)[2]	RR = 1.21 (0.69-2.13)[2]	OR =1.69 (0.94-3.04)[2]
Cross	2010	Prospective cohort study	Colorectal cancer	N_{Cases}=2719 $N_{Controls}$=298229	HR =0.99 (0.87-1.12)[1]	HR=1.19 (1.05-1.34)[1]	HR=1.17 (1.05-1.29)[1]	HR=1.14 (1.01-1.20)[1]

[1]Comparison of highest vs. lowest quintile
[2]Comparison of highest vs. lowest quartile
[3]Comparison of highest vs. lowest tertile
[4]Female-specific study population

2.5.5 Heterocyclic Aromatic, Amines and Colorectal Cancer – Toxicologic Evidence

Dietary exposure to HAAs pose as a potential cancer risk factor in humans because these carcinogenic compounds are produced in relatively high concentrations under normal cooking conditions and their metabolites can covalently bind to DNA resulting in the formation of DNA adducts (66). Left unrepaired, DNA adducts can induce DNA mutations (36). Biologically relevant mutations are a permanent change in DNA that, if unrepaired, may be critical to the survival of the cell (67). Furthermore, a mutation that occurs in the portions of the genome that are involved in regulating processes of cellular growth and differentiation may inactivate tumour suppressor genes or activate proto-oncogenes and initiate the process of carcinogenesis (67).

Both animal and human studies have reported detectable concentrations of HAA-DNA adducts in blood and target site tissues (39-42,68). In animal studies, HAA-DNA adducts form predominately in the liver. In humans, high levels of HAA-DNA adducts generally form in the colon, which may be due to local biotransformation of HAAs and/or inadequate DNA repair occurring in the colon (40).

2.5.6 Biotransformation of Heterocyclic Aromatic Amines

"Biotransformation encompasses all chemical processes of the body that modify endogenous or exogenous chemicals to more water-soluble compounds (67,69)". Generally, biotransformation metabolically converts chemicals in favour of excretion from the body (67,69). However, in contrast, some compounds actually undergo biotransformation to reactive metabolites that are more toxic than the original (parent) compound (67). Biotransformation is affected by factors that pertain to the chemical but host factors including age, sex, existing disease, nutrition status and genetic variability may also affect biotransformation capability (67). These factors contribute to variability in biotransformation by their influence on gene expression

and enzymatic activity. The enzymes for biotransformation are found in many tissues of the body; however the liver has the highest capacity for biotransformation due to its high concentration of enzymes (67). Enzymes catalyzing biotransformation reactions often determine the intensity and duration of action of chemicals and play a key role in chemical carcinogenesis (67).

Bioactivation refers to biotransformation reactions that result in the production of a metabolite that is more toxic than the parent compound (67). Figure 2.2 illustrates the chemical structures of PhIP, MeIQx and DiMeIQx and their DNA adducts. The mutagenicity and, presumably, the carcinogenicity of these HAAs, involve bioactivation by cytochrome P450s (CYP) (Figure 2.3). The exocyclic amine group, common to PhIP, MeIQx and DiMeIQx, undergoes CYP-mediated *N*-oxidation and produces the *N*-hydroxy-HAA species (36). Subsequent conjugation (i.e. modification via the addition of an endogenous chemical) of the *N*-hydroxy-HAA species to *N*-acetoxy-HAA by *N*-acetyltransferases (NATs) or to *N*-sulfonyloxy-HAA by sulfotransferases (SULTs) results in the formation of highly unstable esters that are the ultimate carcinogenic metabolites that react with DNA, resulting in covalent adducts (36,39). Alternate fates of the *N*-hydroxy-HAA species include detoxification by UDP-glucuronsyl transferases (UGTs) or, to a lesser extent, by reduction NADPH reductase (36). As well, reactive metabolites may be detoxified by glutathione S-transferases (GSTs) possibly through a formation of an unstable glutathione conjugate back to its parent amine (36).

Reactive metabolites of HAAs preferentially bind to the carbon-8 position of the guanine residue of DNA resulting in the formation of adducts that tend to be more promutagenic than those bound to other sites on DNA (36). DNA adducts can then either persist or be repaired by nucleotide excision repair (NER) and possibly, to a lesser extent, base excision repair (BER)

(70). Therefore, the steady-state level of DNA adducts reflects the balance between exposure to a carcinogen, adduct formation and adduct elimination by DNA repair.

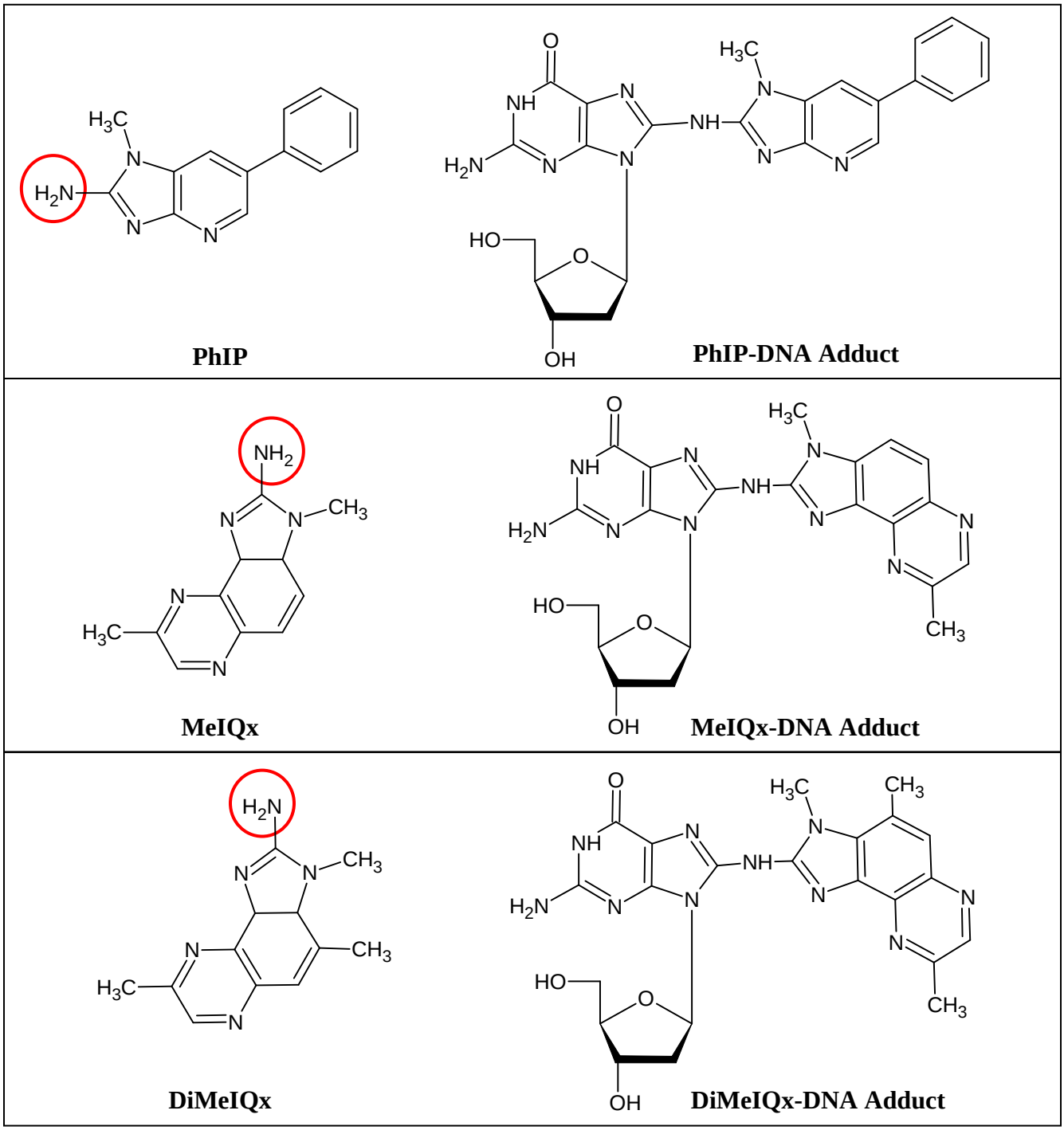

Figure 2.2: Chemical structures of PhIP, MeIQx and DiMeIQx with the exocyclic amine group circled in red and their DNA adducts (6).

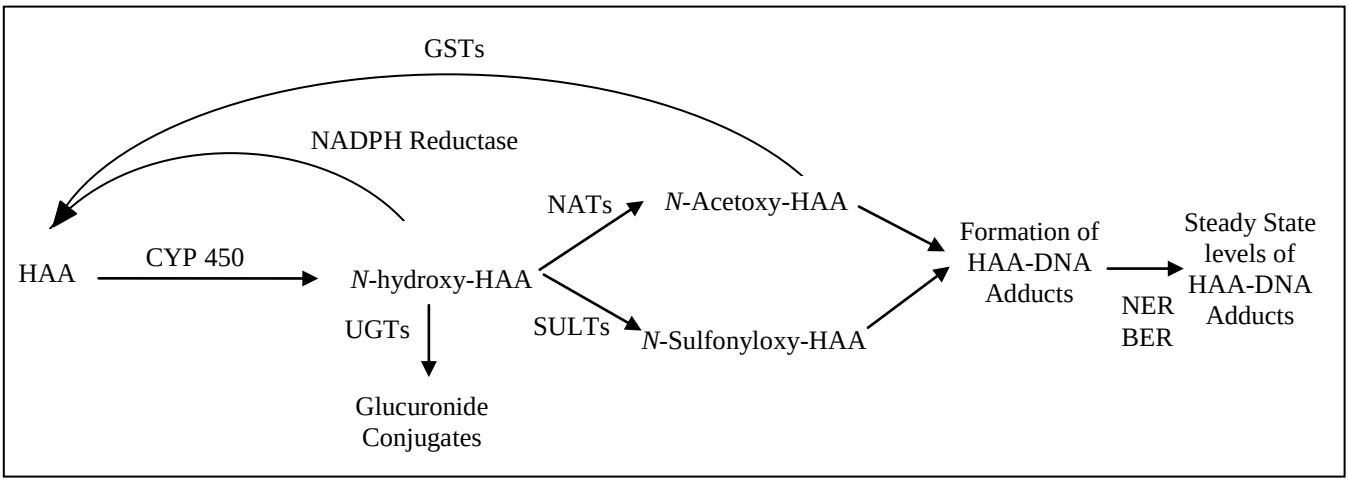

Figure 2.3: Major pathway of metabolism of HAA (PhIP) in humans (6).

2.5.7 Heterocyclic Aromatic Amine-DNA Adducts – Relevance to Colorectal Cancer

Experimental studies have observed that during chronic carcinogen exposure, levels of carcinogen-DNA adducts in target tissues are dose-related to exposure and could potentially be predictive of human disease risk (71).

In humans, there are two ways that HAAs and/or their metabolites can reach the colon and initiate the carcinogenic process through the formation of DNA adducts: 1) via luminal absorption of dietary HAAs from the digestive tract directly (1a) or luminal absorption of HAA metabolites transported from the liver to the colon through bile (1b); and 2) via blood circulation (2) (Figure 2.4, Part A) (25).

The human colon is characterized by crypts that are approximately 50 cells deep; the stem cells (i.e. cells that have the capacity to differentiate into many distinct cell types) reside at the base of the crypt where they have some protection from the mutagenic environment of the colon lumen (Figure 2.4, Part B). The structure of the crypt and the dynamics of cell proliferation ensure that under normal circumstances stem cells replicate in the lower one third of the crypt and the progeny migrate upwards to form cells that line the upper two thirds of the

crypt. By the time normal crypt cells reach the surface they are no longer replicating, they are differentiated and may be beginning to undergo apoptosis (i.e. programmed cell death) (25).

Once HAAs/HAA metabolites reach the colon via the digestive tract and/or the circulation, enzymes in mucosal cells and in colon bacteria may bioactivate HAAs or their metabolites to produce the ultimate carcinogenic metabolite. These reactive metabolites may react with DNA of stems cells or other cells lining the crypts to form DNA adducts (25). In general, adducts that remain unrepaired and thus persist, can cause mutational events in regions of DNA that are involved in regulating processes of cellular growth and differentiation which can initiate the process of carcinogenesis (71-73).

i. Top-down Versus Bottom-up Hypothesis:

It is unknown whether the carcinogenicity of HAAs is a consequence of luminal (top-down) or blood delivery (bottom-up). The bottom-up hypothesis proposes that the first mutation occurs in stem cells as a result of interaction with blood-borne mutagens (e.g. DNA adduct formation in stem cells) (Figure 2.4, Pathway 2). This interaction can result in replicating abnormal cells that migrate upwards producing a polyp at the top of the crypt (74). The polyp would then be exposed to the genotoxic contents of the luminal stream and, consequently, increase the likelihood of further mutations (74).

In contrast, the top-down hypothesis put forth by Shih et al. proposes that the development of adenomas begins with the genetic alteration of cells that line the crypt (e.g. DNA adduct formation in cells that line the top of the crypt) and would then spread laterally and downward to form new crypts that would eventually replace the preexisting normal crypts (Figure 2.4, Pathway 1a and 1b) (75). The top-down hypothesis is supported by research that examined the molecular characteristics of cells from the top and bottom of the same crypt in

small colorectal adenomas (75). Specifically, it was observed that dyplastic or abnormal cells at the top of crypts frequently exhibited genetic alteration and neoplasia-associated patterns of gene expression, whereas those at the bottom of the same crypt did not display these alterations (75). Both hypotheses are considered controversial and it is recognized that further research in adenoma formation is required to advance the understanding of CRC initiation via exposure to genotoxicants.

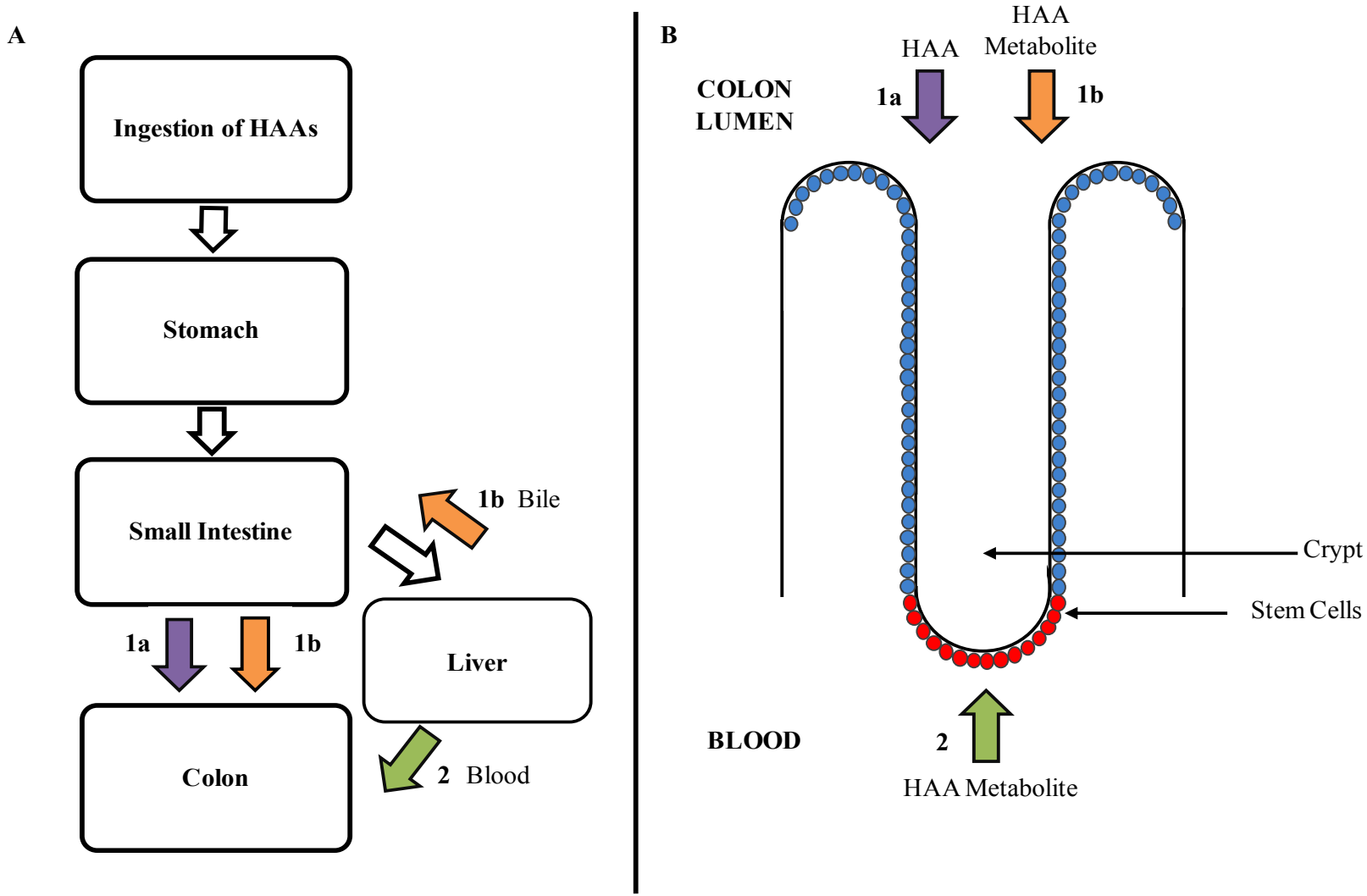

Figure 2.4: A) Schematic of HAA transport in the gastro-intestinal tract. B) Interaction of colon crypt cells with luminal or blood-borne HAA metabolites. In both A and B: 1a) illustrates the luminal absorption of HAAs from food in the digestive tract (orange arrow); 1b) illustrates the luminal absorption of HAA metabolites from bile (purple arrow) and; 2) illustrates the absorption of HAA metabolites from blood (green arrow).

2.6 Heterocyclic Aromatic Amines and Colorectal Adenoma Risk – A Molecular Epidemiologic Approach

Phase 1 of this doctoral dissertation conceptualized DNA adducts as a biomarker of biologically effective dose to HAAs and investigated the relationship between dietary exposure to HAAs and the levels of DNA adducts in blood leukocytes. An understanding of the relationship between dietary exposure to HAAs and DNA adducts contributes to the assessment of the potential role of HAAs in carcinogenesis.

The use of a biomarker of HAAs in epidemiologic research has considerable potential for more accurate quantification of exposure to HAAs and to inform on their mechanisms of action. However, to achieve accurate estimates of risk in molecular epidemiologic studies, the selection of a biomarker requires consideration of: 1) the appropriate method for biomarker measurement; 2) validity and reliability of the biomarker measurement method; 3) the selection of the biologic matrix used to measure the biomarker; 4) within-subject variability and the implications on the time-frame of exposure representation and; 5) a critical appraisal of the current literature on the determinants of the biomarker, the relationship between exposure and the biomarker of interest and the association between biomarker and the ultimate outcome of interest (76).

2.6.1 DNA Adducts – Measurement Methods

In this doctoral dissertation, the identification and quantification of specific HAA-DNA (i.e. PhIP-DNA and MeIQx-DNA) adducts using mass spectrometry (MS) was initially proposed. However, after extensive method development and validation, the detection and quantification of specific HAA-DNA adducts using MS in human blood leukocytes was deemed infeasible; a summary of the method development and validation of this approach is provided in Appendix 1. In place of specific HAA-DNA adduct measures, this dissertation utilized the ^{32}P-

postlabelling method to quantify bulky DNA adducts as a proxy for HAA-DNA adduct levels in Phase 1.

i. Mass Spectrometry Versus ^{32}P-Postlabelling

MS is an analytical technique that can be used to identify and quantify the amount of a given compound (PhIP-DNA and MeIQx-DNA adducts) in a complex sample (human biologic media i.e. blood) (77). The great advantage of MS for the study in question is that it can identify and quantify specific carcinogen-DNA adduct levels (78); however, very few population-based study have utilized MS to quantify HAA-DNA adducts as the method requires large amounts of DNA, is labour intensive and validity concerns remain.

The ^{32}P-postlabelling method is the most widely applied and sensitive procedure for the quantification of DNA adducts (78). This technique was first described in 1981 by Randerath et al. (79) and has since undergone several refinements to enhance sensitivity and specificity. Briefly, analysis involves enzymatic digestion of DNA to nucleotides, selective radio-labelling of carcinogen-bound nucleotides, separation of DNA adducts by thin-layer chromatography and detection of adducts by measuring radioactivity (80). However, the key disadvantage of this assay is that it is non-specific; that is, the technique alone only provides an overall measure of total bulky DNA adducts in a sample. Though the measurement of total bulky DNA adducts is applicable to a wide variety of DNA adduct structures, the contribution of specific HAA-DNA adducts (i.e. PhIP-DNA and MeIQx-DNA adducts) to the overall quantity of DNA adducts cannot be determined. Nevertheless, bulky DNA adducts have been utilized in the literature as a marker of both exposure to a genotoxic compound and a marker of early biologic effect; specifically, observational studies are increasingly employing the ^{32}P-postlabelling method to assess the relationship between bulky DNA adducts and CRC risk (72,81-83). The ^{32}P-

postlabelling method employed in Phase 1 detected bulky DNA adducts as a whole, which would encompass HAA-derived adducts and also other bulky lesions.

2.6.2 DNA Adducts – Validity and Reliability

A degree of error is intrinsic to any measurement process (84). Validity in the context of biomarker measurement refers to the ability of the assay to accurately measure the marker of interest (76). Reliability, on the other hand, describes the extent to which "results agree when obtained by different observers, study instruments or procedures or by the same observer, study instrument or procedure at different points in time (85)". In biomarker development and validation, both validity and reliability are critical. However, since validity is not often measureable, reliability is often used as a surrogate. Reliability or biomarker variability can be influenced by: 1) within-subject variability (to be discussed in Section 2.6.4) and 2) the imprecision of the observer. Variability due to the imprecision of the observer can be classified into within-observer variability or between-observer variability. Within-observer variability describes the variability in the assay determination conducted twice on the same sample by the same technician using the same technique. In contrast, between-observer variability defines variability in the assay determination conducted on the same sample by two (or more) technicians using the same assay (85). Measures of reliability assessed in this research are further detailed in Chapter 4 Section 4.1.5.

The ^{32}P-postlabelling method is a valid and established method for the measurement of bulky DNA adducts (86). In 1999, an international project led by D.H. Phillips and M. Castegnaro standardized the protocols for the ^{32}P-postlabelling method. This project involved a critical appraisal of all aspects of the ^{32}P-postlabelling procedure, including the establishment of validity, and investigations to examine the influence of a number of key variations that

contribute to the reliability of the assay (86). This project led to a consensus protocol in a first inter-laboratory trial involving 25 participants in Europe and the USA. As a result of this project a recommended set of procedures has been developed for the valid and reliable detection and quantification of DNA adducts formed by HAAs, PAHs and other agents (86).

2.6.3 DNA Adducts – Selection of Biologic Matrix

Theoretically, any tissue can be used for the quantification of DNA adducts. However, tissues of established interest for monitoring human exposure to genotoxicants like HAAs include oral mucosa, blood (leukocytes and lymphocytes) and target site tissues like breast and colon tissue (87). Guidance for the selection of the biological matrix used for DNA adduct analysis includes consideration of the metabolism and pharmacokinetics of the analyte of interest and whether the selected matrix either includes the target cells of toxiciological relevance or could be used as a surrogate for target cell concentrations (87).

In the conduct of epidemiologic research among healthy participants, accessible biological matrices such as oral mucosa or blood are most frequently employed since the more relevant target tissues (e.g. colon mucosa) are not usually available (76). However, DNA adduct quantification requires large amounts of DNA that may be beyond the yields available from an oral mucosa sample. Therefore, measurement of DNA adducts in fasting blood leukocytes was considered to be most applicable to Phase 1 of this research which aimed to understand the relationship between dietary exposure to HAAs and bulky DNA adducts in a healthy population. The use of blood leukocytes as the biological matrix to measure DNA adducts is supported by previous investigations (discussed in Section 2.6.6). Also, relatively high DNA yields given a reasonable volume of blood and the potential for blood measures to be a potential surrogate for

colon tissue concentrations, given the bottom-up hypothesis discussed in Section 2.5.7, support the use of DNA derived from blood leukocytes for adduct measurement.

2.6.4 DNA Adducts – Within-subject Variability and Timeframe of Exposure Representation

In Phase 1 of this research, the relationship between dietary exposure to HAAs and levels of bulky DNA adducts in blood leukocytes was investigated in a cross-sectional study. Thus, the utilization of bulky DNA adducts as biomarkers of HAA levels required consideration of the within-subject variability to understand what time-frame was represented by a cross-sectional (one-time) measure of bulky DNA adducts. The major components considered included: 1) the short-term variability of DNA adduct levels; 2) the long-term variability of DNA adduct levels introduced by cell turnover and; 3) the long-term stability of dietary habits.

i. Short-Term Variability of DNA Adducts

Data on the short-term variability of bulky DNA adducts is limited for blood leukocytes. In an experimental study of PhIP-DNA adduct formation in blood leukocytes as measured by MS, the formation of specific PhIP-DNA adducts (over 24 hours) was shown to fluctuate after an acute administered exposure but adducts were found to return after 12 hours to steady-state levels that may be more reflective of habitual exposures (39). Specifically, PhIP-DNA adducts increased gradually with time after ingestion of a diet-relevant dose of PhIP encased in a gelatin capsule, reached a plateau two to four hours after exposure and finally reached steady state 12 hours after exposure (88). Thus, after a 12-hour fast, levels of DNA adducts are believed to reflect steady state concentrations.

ii. Long-Term Variability of DNA Adducts Introduced by Cell Turnover

The long-term variability of bulky DNA adduct levels in blood leukocytes was

hypothesized to be a function of the rates of cell turnover. White blood cells or leukocytes consist of different and diverse types of cells including neutrophils, eosinophils, basophils, lymphocytes and monocytes (89). Neutrophils are the most predominant of all the white blood cells comprising 50 to 70%, followed by lymphocytes (20-30%), monocytes (2-8%), eosinophils (2-4%) and basophils (<1%) (89). Most leukocytes have a very short life span, typically between a few days and a week, with the exception of lymphocytes which have a mean age of 100 to 200 days (90). It is recognized that bulky DNA adducts in blood lymphocytes would represent the ideal measure of long-term exposure to dietary HAAs. However, this would require large volumes of blood (approximately two times the amount required for DNA isolated from blood leukocytes) limiting its feasibility in the conduct of research in a healthy population.

iii. Long-Term Stability of Dietary Habits

Repeated assessments of dietary habits are better equipped to capture habitual dietary intakes than a cross-sectional assessment. However, in a study of repeatability of dietary data over a seven-year period, it was found among subjects with no reported dietary change that correlations between assessments at baseline and seven years later were 0.63 or higher for most macronutrients (protein, fat and carbohydrate) (91). Similar findings have been reported by Goldbohm et al. and Kimokoti et al., over a five- and eight-year period, respectively (92,93). Collectively, results from these studies concluded that dietary patterns change minimally over time and substantiates that a single FFQ administration is not only relevant to the dietary habits in the period of evaluation but may extend to a much longer period (91-93).

2.6.5 Determinants of Bulky DNA Adducts Measured by ^{32}P-Postlabelling – Epidemiologic Evidence

The ^{32}P-postlabelling method employed in Phase 1 detects general bulky DNA adducts in white blood cells which would encompass HAA-derived adducts and also other bulky lesions such as PAH-DNA adducts. Factors that have been reported to affect bulky DNA adduct levels in blood includes smoking, diet (fruit and vegetable consumption), BMI, genetic polymorphisms, seasonality and air pollution (94). In a pooled analysis of 11 study cohorts encompassing 3,600 participants, the effect of age, gender, smoking, BMI, seasonality and genetic polymorphisms on bulky DNA adducts was investigated (94). Bulky DNA adducts were measured in human white blood cells with similar ^{32}P-postlabelling techniques; results indicated that bulky DNA adducts were associated with seasonality (with higher adduct levels in winter) and authors postulated that air pollution may partly explain the relationship. In addition, current and former smokers had lower levels of DNA adducts than never smokers and a non-significant decrease in DNA adducts was found with increasing age ($p=0.09$). No associations were observed for BMI and the genetic polymorphisms under investigation (CYP1A1, GSTM1, GSTT1, myeloperoxidase and NAD(P)H:quinone oxidoreductase) (94).

2.6.6 Meat Consumption and DNA Adduct – Epidemiologic Evidence

As discussed in Section 2.6.1, there has been an absence of analytical methods with sufficient sensitivity for quantifying concentrations of specific HAA-DNA adducts. This has hindered the use of HAA-DNA adducts as biomarkers of HAAs. To date, no observational study has investigated the association between dietary HAAs and specific HAA-DNA adduct levels in healthy individuals. A study conducted by Magagnotti et al., utilized a MS method to investigate the association between meat consumption and PhIP-DNA adducts levels in blood lymphocytes

among 76 CRC patients. PhIP-DNA adduct levels tended to be higher in individuals consuming greater than the median number of servings of meat per week but the difference was not found to be statistically significant (41).

Two cross-sectional studies have evaluated the association between meat consumption and bulky DNA adducts as measured by the ^{32}P-postlabelling technique among healthy individuals (95,96). One study investigated the association between dietary and lifestyle factors, and bulky DNA adducts in blood leukocytes among 296 healthy Spanish volunteers; meat intake was found to be associated with a non-significant increase in adduct levels (97). Another study investigated the role of dietary and genetic factors on leukocytic bulky DNA adduct levels among 634 healthy Italian volunteers and reported a significant interaction between GSTM1 polymorphism and white meat consumption though no main effects of red or white meat intake were found (96). Limitations of these studies included a limited number of meat items considered in the dietary assessment and the lack of assessment of specific dietary HAA intake.

2.6.7 DNA Adduct and Colorectal Cancer Risk –Epidemiologic Evidence

Research on the association between specific DNA adducts and CRC/colorectal adenoma risk is lacking. However, one case-cohort study of 450 subjects investigated the association between bulky DNA adducts in blood leukocytes and CRC risk. This study utilized a ^{32}P-postlabelling technique to quantify bulky DNA adducts and a RR of 2.29 (95% CI: 1.05-5.00) for risk of CRC was reported when comparing the highest versus lowest tertiles of adduct levels (98). In addition, three small studies have examined the relationship between CRC risk and the level of bulky DNA adducts (measured by ^{32}P-postlabelling technique) in colorectal mucosa (72,82,83). Pfohl-Leszkowicz et al. (72) reported a significantly higher mean total DNA adduct level in normal-appearing colon tissue biopsies from 18 colon cancer cases as compared to 19

healthy control patients. In contrast, Jonsson et al. (83) compared total DNA adduct levels in normal-appearing colon tissue among 10 healthy patients, 22 adenoma patients and 32 colon cancer patients and reported no differences across study groups. Finally, the comparison of DNA adduct levels in 24 cancerous tissues samples from colon cancer patients and 19 normal tissue samples obtained from hospital controls reported a non-significant increase in colon cancer risk when contrasting levels of DNA adducts in cancerous tissue versus normal tissue from healthy patients (82). Though the measurement of bulky DNA adducts in target site colorectal tissues is ideal, an obvious limitation of these studies is a small sample size.

2.7 Conclusions and Perspectives

In summary, this review highlights the complexities inherent in epidemiologic research in investigating the role of environmental factors like HAAs in cancer development. Overall, though studies examining the effect of high meat consumption on CRC risk have produced some inconsistent results, the weight of the evidence points towards an elevated risk. Exposure to meat-derived carcinogens like HAAs is an emerging mechanism linking meat consumption with CRC development. In this review, the mutagenicity and genotoxicity of HAAs is introduced through the formation of DNA adducts. A prevailing theme of this review is that it is difficult to establish the relationship between meat-derived carcinogens and CRC risk using traditional epidemiologic methods, primarily due to the long latency between exposure and outcome, exposure misclassification and potential modification of the relationship via individual susceptibility. In Phase 1 of this dissertation, using molecular epidemiologic methods, the relationship between dietary exposure to HAAs, individual susceptibility and the formation of bulky DNA adducts was examined. A discussion on biomarker validity related to the use of DNA adducts as a biomarker of HAA exposure and/or early biologic effect was presented.

Finally, Phase 2 of this research investigated the roles of dietary HAA and meat mutagenicity exposures on colorectal adenoma development. The evaluation of individual susceptibility to the carcinogenic effects of HAAs is central to the contribution of Phase 2; the role of individual susceptibility in the HAA-CRC continuum conceptualized in this dissertation will be further reviewed in Chapter 3.

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Chapter 3: Literature Review

Effect Modification in the Heterocyclic Aromatic Amine-Colorectal Cancer Relationship

There are considerable inter-individual differences in susceptibility to environmental toxicants. Variants in genes encoding enzymes involved in the biotransformation (bioactivation and detoxification) of heterocyclic aromatic amines (HAAs) and DNA repair may all play roles in determining an individual's susceptibility to HAAs and their subsequent risk of CRC. Research that incorporates the role of individual susceptibility to the investigation of the HAA-CRC relationship will have the potential to contribute to the understanding of underlying mechanisms, and may clarify and strengthen the evidence for a causal relationship. This Chapter presents the strategies used to identify and select polymorphisms in genes of interest for Phase 1 and 2. Relevant details of the strategies used in each Phase are described separately.

3.1 The Investigation of Gene-Environment Interactions in the HAA-CRC Relationship

Gene-environment interactions are of interest to the HAA-CRC relationship because individuals who carry polymorphisms (i.e. alternate forms of a gene found at frequencies greater than 1% of the population (1)) which confer differences in biotransformation (i.e. higher bioactivation and lower detoxification) and/or impaired or sub-optimal DNA repair activity may be most susceptible to the carcinogenic effects of HAAs. As a result, the strongest risk estimates associated with dietary HAA exposure on DNA adduct levels (Phase 1 outcome of interest) and colorectal adenoma risk (Phase 2 outcome of interest) are expected in the genetically susceptible sub-population. Examination of gene-environment interactions is crucial since a larger risk estimate (associated with the environmental factor – dietary HAAs) seen in the genetically susceptible sub-group provides a stronger argument for causality and informs on the underlying biologic mechanisms of HAAs in carcinogenesis.

Gene-environment interactions along the continuum of events underlying dietary HAA exposure and CRC development have been investigated by selecting polymorphisms hypothesized to affect genes involved in HAA biotransformation (2-5), and/or genes involved in DNA repair (either repair of adducts or damage caused by HAAs/DNA adducts) (6-12). However, previous research rarely stated or detailed the selection strategies that were employed to identify the polymorphisms of interest. Accordingly, separate gene selection strategies were developed and utilized in Phase 1 and 2 of this doctoral dissertation. In general, genes with the ability to determine DNA adducts (Phase 1 outcome), including genes involved in the biotransformation of HAAs and repair of DNA adducts, were of interest to Phase 1. For Phase 2, genes with the evidence to support involvement in the biotransformation of HAAs and DNA repair (repair of DNA adducts and damaged caused by HAAs/DNA adducts) which would thus determine susceptibility to colorectal adenomas (Phase 2 outcome) were of interest.

3.2 Epidemiologic Principles Underlying Gene Selection Strategies

In the selection of genetic variants of interest to Phase 1 and 2, a general schema was developed for prioritization to ensure the selection of polymorphisms with the strongest biologic plausibility and high exposure prevalence. This general approach would enable the planning and conduct of research with adequate statistical power, and the interpretation and translation of research findings with meaningful population impact.

Briefly, to select genes with the strongest biologic plausibility for Phase 1 and 2, relevant genes of interest from a mechanistic perspective were considered as those involved in the biotransformation of HAAs (of interest to Phase 1 and 2), repair of DNA adducts (of interest to Phase 1 and 2) and repair of DNA damage induced by HAAs (of interest to Phase 2 only since DNA damage was conceptualized as a downstream consequence of DNA adducts). Functional

polymorphisms in these identified genes were next reviewed; a functional polymorphism was defined as those which have demonstrated effects on gene expression or enzyme activity *in vitro/in vivo.* It follows that since the identified genes are involved in the various mechanisms relevant to Phase 1 and 2, polymorphisms that confer a functional effect would be candidate polymorphisms of interest for the investigation of gene-environment interactions.

An allele is one of a number of alternate forms of a gene and the frequency of alleles in a population can be used to predict the frequency of the corresponding genotypes. Candidate polymorphisms were prioritized based on allele frequency. Specifically, only polymorphisms with a minor allele frequency (MAF) greater than 10% in Caucasians were prioritized; that is, the frequency at which the less common allele occurs must be greater than 10%. A single nucleotide polymorphism (SNP), the most common type of polymorphism, describes a single nucleotide being replaced with another, and has two alleles that determine the SNP genotype which could be either homozygous wildtype (i.e. two of the more common allele), heterozygous (i.e. one each of the more and less common allele) or homozygous variant (i.e. two of the less common allele). Under Hardy-Weinberg Equilibrium, a state in which genotype frequencies do not change from one generation to the next in a population, for "two alleles P and Q, at frequencies p and q respectively, homozygotes for P are found at frequency p^2, homozygotes for Q are found at frequency q^2 and heterozygotes are found at frequency 2pq (13)".

The following section illustrates the relationships between MAF, genotype distribution and population attributable fraction (PAF) under Hardy-Weinberg Equilibrium. For gene X with a functional SNP that changes cytosine (C) to thymine (T) at nucleotide position Y, a MAF of 10% describes the frequency of allele 1 containing nucleotide T at position Y (the less common allele) and accordingly the frequency of allele 2 containing nucleotide C is 90% (Table 3.1).

Table 3.1. Genotype categories associated with allele C and T and their associated frequencies

Genotypes	Allele 1 (T)	Allele 2 (C)	Frequency
Allele 1 (T)	CC	CT	0.10
Allele 2 (C)	CC	TT	0.90
	0.10	0.90	1.00

Under Hardy-Weinberg Equilibrium, we expect the following genotype distributions for CC, CT and TT to be 0.81, 0.18 and 0.01, respectively (calculations are outlined in Table 3.2).

Table 3.2. Expected frequency of genotype categories associated with allele C and T under Hardy-Weinberg Equilibrium

Genotype Frequency	Allele 1 (T)	Allele 2 (C)	Frequency
Allele 1 (T)	(0.10)*(0.10)/1.00 = 0.01	(0.90)*(0.10)/1.00 = 0.09	0.10
Allele 2 (C)	(0.90)*(0.10)/1.00 = 0.09	(0.90)*(0.90)/1.00 = 0.81	0.90
	0.10	0.90	1.00

To ensure that polymorphisms of interest have a meaningful population impact, Table 3.3 displays the PAF based on the joint effect of gene and environmental factors on the outcome of interest. Note that calculations are based on dichotomizing the environmental factor at the median (yielding 50% of the population categorized as exposed) and categorization of the polymorphism into an 'at risk' vs. 'not at risk genotype.' Specifically, heterozygotes can be grouped with either the homozygote wildtype or homozygote variant categories; selection based on functional evidence supporting a certain categorization is ideal but often sample size is the driving factor in determining groupings. In this example, heterozygotes were grouped with the homozygote variant category; as well, this calculation assumes that the relative risk (RR) for the environmental exposure among the 'not at risk' genotype is null. Table 3.3 displays the PAFs associated with having the 'at risk' genotype and the environmental exposure across varying relative risk (RR) estimates and prevalence of the 'at risk' genetic factor. With a MAF of 10%, a PAF of greater than 10% is only achieved if the RR is 2.5. With increasing MAF, PAF is greater than 10% at RR=2.0 (when MAF $\geq$ 15%) and RR=1.5 (when MAF $\geq$ 25%).

Table 3.3. Population attributable fraction calculated as a function of the joint effect of gene and environmental factors with varying MAF and RR estimates Shaded cells indicated PAR less than 10%.

MAF	Prevalence of 'At Risk' Genotype[1]	Prevalence of 'At Risk' Genotype + Exposed[2]	Population Attributable Fraction[3]		
			RR=1.5	RR=2.0	RR=2.5
5%	0.10	0.05	2.0%	5.0%	7.0%
10%	0.19	0.10	5.0%	9.0%	12.0%
15%	0.28	0.14	6.0%	12.0%	17.0%
20%	0.36	0.18	8.0%	15.0%	21.0%
25%	0.44	0.22	10.0%	18.0%	25.0%
30%	0.51	0.26	11.0%	20.0%	28.0%
35%	0.58	0.29	13.0%	22.0%	30.0%
40%	0.64	0.32	14.0%	24.0%	32.0%
45%	0.70	0.35	15.0%	26.0%	34.0%
50%	0.75	0.38	16.0%	27.0%	36.0%

[1]Heterozygotes are grouped with homozygote variants
[2]Exposure is dichotomized at the median
[3]PAR assumes a RR of 1.0 for the environmental factor in the 'not at risk' genotype.

Numerous polymorphisms are found in genes involved in the relevant mechanisms under investigation in Phase 1 and 2. However, with the consideration of the epidemiologic principles underlying exposure prevalence including statistical power and meaningful population impact, only polymorphisms with a MAF of 10% were prioritized in Phase 1 and 2. Specific details of the gene selection strategies used for Phase 1 and 2 are outlined in Section 3.3 and 3.4.

3.3 Phase 1: Dietary HAA Intake and Bulky DNA Adducts

The relationship between dietary HAA intake and the steady-state level of bulky DNA adducts measured in blood leukocytes of 125 healthy volunteers was investigated in Phase 1. Variation in genes involved in the biotransformation (bioactivation and detoxification) of HAAs, and the repair of DNA adducts was of interest as it was hypothesized that the association between dietary HAA intake and bulky DNA adduct levels is modified by differences in genetic susceptibility (Figure 3.1). Specifically, the relationship between steady-state levels of the biologically effective dose of HAAs given an administered dose of HAAs was hypothesized to

vary according to different forms of a gene encoding enzymes which are involved in the bioactivation and detoxification of HAAs, and in DNA repair mechanisms responsible for the repair of DNA adducts (Figure 3.1).

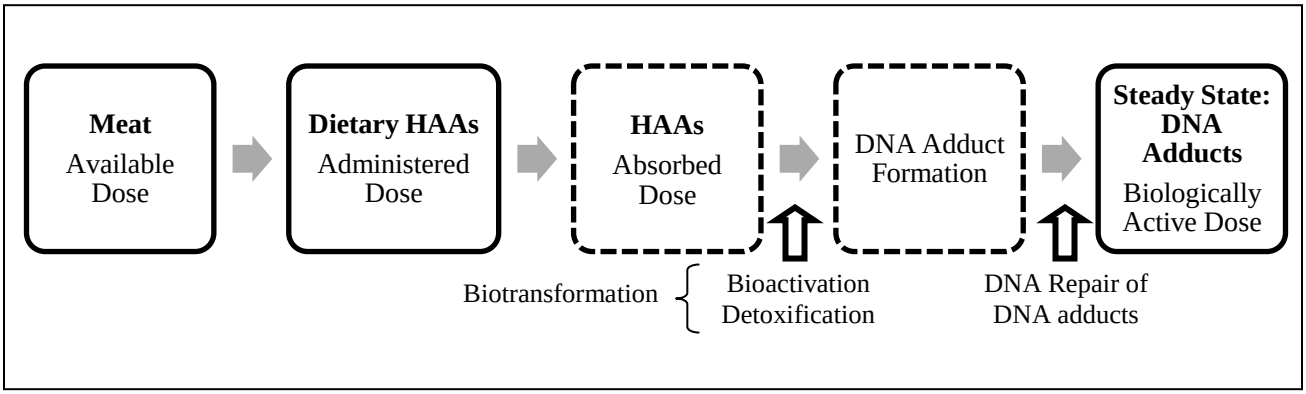

Figure 3.1: Conceptual model of Phase 1; genes of interest include those involved in the biotransformation of HAAs and the repair of DNA adducts. Solid lines refer to measured variables of interest to Phase 1.

3.3.1 Phase 1: Gene and Polymorphism Selection Strategy

The biotransformation of HAAs was described in 2.5.6. Genes of interest were first identified based on biologic plausibility. A review of toxicologic evidence outlining the specific genes involved in the biotransformation of HAAs and the repair of DNA adducts in humans was conducted. Note that the literature surrounding the biotransformation of HAAs mostly utilizes PhIP as the HAA of interest therefore the applicability to MeIQx and DiMeIQx metabolism may need to be considered (6-12,14-17). Genes identified by this initial literature review are outlined in Table 3.4. Candidate polymorphisms within these genes were identified through established nomenclature websites when available (cytochrome P450 (CYP) 1A1, CYP1A2, CYP1B1, *N*-acetyltransferase (NAT) 1, and NAT2) and when unavailable literature reviews were conducted (sulfotransferase (SULT) 1A1, glutathione S-transferase (GST) A1, UDP-glucuronsyl transferase (UGT) 1A, xeroderma pigmentosum group A (XPA), xeroderma pigmentosum group C (XPC), xeroderma pigmentosum group D (XPD), excision repair cross-complementing group 1

(ERCC1), and x-ray repair cross-complementing group 1 (XRCC1)). Candidate polymorphisms in specific genes were only considered if their MAF was greater than 10%. Further prioritization of genes involved in the biotransformation of HAA, and in repair of DNA adducts is outlined in the following subsections; note that a different set of prioritization criteria was employed for genes involved in the bioactivation and detoxification of HAAs versus genes relevant to the repair of DNA adducts.

Table 3.4. Genetic factors of interest for Phase 1

Mechanisms	Genes of Interest
Bioactivation of HAAs	CYP1A1, CYP1A2, CYP1B, NAT1, NAT2, SULT1A1
Detoxification of HAAs	GSTA1, UGT1A1
Repair of DNA Adducts	XPA, XPC, XPD, ERCC1, XRCC1

i. Polymorphisms in Genes Involved in the Biotransformation of HAAs

For genes involved in biotransformation of HAAs, a set of standardized polymorphism selection and prioritization strategies was used. Specifically, since the level of DNA adducts in blood leukocytes is the outcome of interest for Phase 1, priority was first given to polymorphisms with demonstrated effects on HAA-DNA or bulky DNA adducts levels in blood and other biologic media. Then, priority was next given to polymorphisms with demonstrated effects on HAAs in urine. This criterion was only applied to prioritize genes involved in the bioactivation and detoxification of HAAs and not genes involved in the repair of DNA adducts as HAAs in urine are not anticipated to be affected by variation in DNA repair activity. Finally, polymorphisms with functional evidence demonstrating an effect of the polymorphism on enzyme activity in *vitro/in vivo* were given the next priority.

ii. Polymorphisms in Genes Involved in the Repair of DNA Adducts

For genes involved in the repair of DNA adducts identified in the literature, priority was first given to polymorphisms with demonstrated effects on HAA-DNA or bulky DNA adducts.

Then, polymorphisms with functional evidence demonstrating the effect of the polymorphism on enzyme activity in *vitro/in vivo* were given the next priority. Figure 3.2 outlines a schematic illustrating the gene selection strategy employed in Phase 1. A summary table that prioritizes the polymorphisms of interest to Phase 1 using the lines of evidence described is given in Table 3.5.

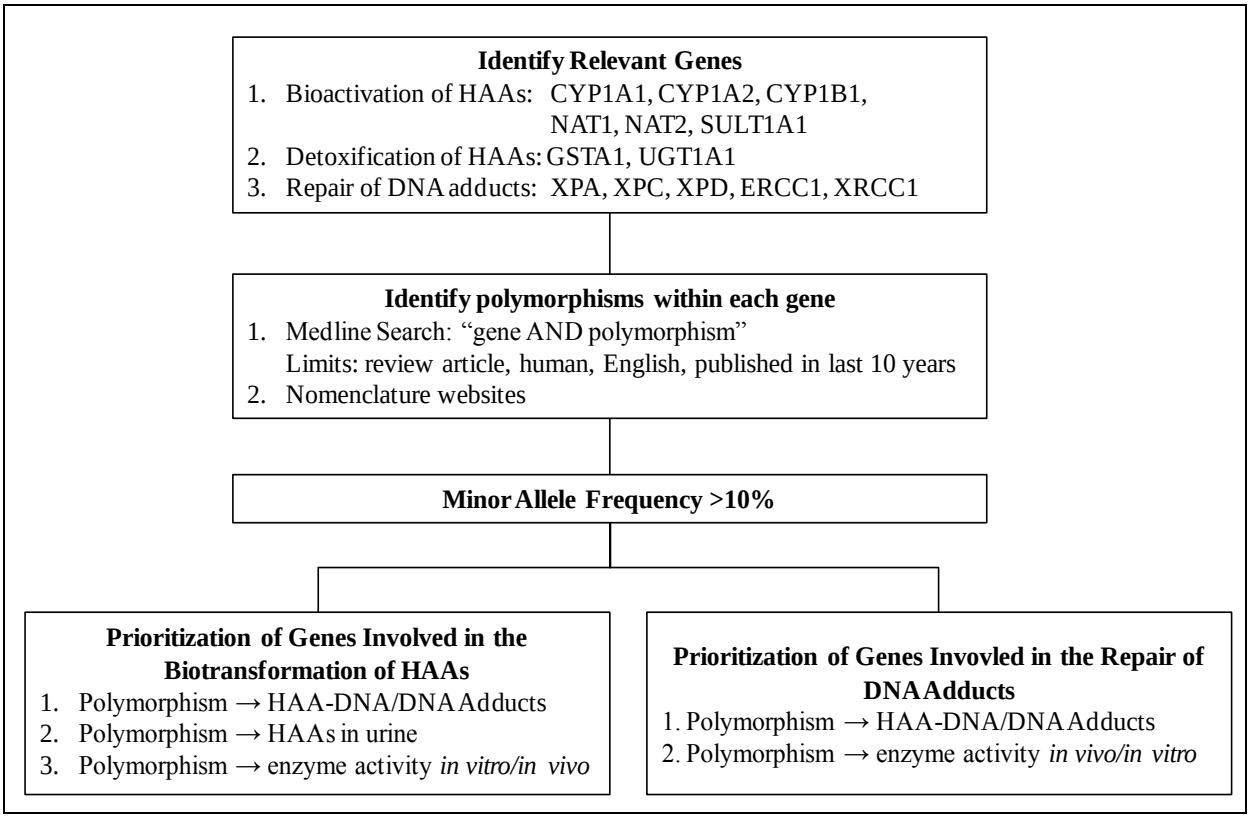

Figure 3.2: Schematic of Phase 1 gene selection strategy

Table 3.5. Summary table of polymorphisms of interest to Phase 1. Unshaded rows indicate the polymorphisms selected for genotyping in Phase 1.

Gene	Allele	MAF	Nucleotide Changes (rs[1])	Evidence on DNA Adducts[2]	Evidence on Urinary HAAs[2]	Functional Evidence - In vitro/vivo[2]
CYP1A1	CYP1A1*2A	25%	3798T>C (rs4646903)	No	Yes	Yes
CYP1A2	CYP1A2*1F	35%	−163C>A (rs762551)	No	Yes/No	Yes
CYP1B1	CYP1B1*2	-	142C>G; 355G>T (rs10012; rs1056827)	Yes	N/A	No
NAT1	NAT1*10	-	1088T>A; 1095C>A (rs1507126, rs15561)	Yes	N/A	Yes
NAT2	NAT2*5K	-	282C>T; 341T>C (rs1041983; rs1801280)	Yes	Yes/No	Yes
GST	GSTA1*B	-	−69C>T (rs3957356)	Yes	N/A	Yes/No
XPA	-	40%	−4G>A (rs1800975)	Yes	-	Yes
XPD	-	27%	2251A>C (rs13181)	Yes	-	Yes
XPD	-	41%	934G>A (rs1799793)	Yes	-	Yes
XRCC1	-	47%	1196A>G (rs25487)	Yes	-	Yes
CYP1A2	CYP1A2*1B	32%	5347T>C (rs2470890)	N/A	No	No
CYP1A2	CYP1A2*1D	24%	−2467delT (35694136)	N/A	N/A	No
CYP1B1	CYP1B1*4	19%	4390A>G (rs1800440)	No	N/A	Yes
CYP1B1	CYP1B1*3	39%	4326C>G (rs1056836)	No	N/A	N/A
SULT1	SULT1A1*2	34%	638G>A (rs9282861)	No	N/A	Yes
UGT1A1	UGT1A1*28	47%	(TA)(7) (rs8175347)	N/A	N/A	Yes
XPC	-	34%	2704C>A (rs2228001)	No	-	Yes
ERCC1	-	36%	354T>C (rs11615)	No	-	N/A

[1]Reference SNP identification number
[2] -: Not relevant; N/A: No study identified; Yes/No: Conflicting evidence

3.3.2 Phase 1: Polymorphisms in Genes Involved in Biotransformation of HAAs

The following subsections are organized according to genes involved in the bioactivation (CYP1A1, CYP1A2, CYP1B1, NAT1, NAT2 and SULT1A1) and detoxification of HAAs (UGT1A1 and GSTA1). Within each subsection a brief description of the relevant genes and their role in HAA biotransformation is described. Then, a more detailed description of the specific polymorphism within each class of genes is presented and polymorphisms are subsequently ranked in order of priority as outlined in Table 3.5. In the selection of polymorphisms in genes involved in the biotransformation of HAAs, genes expressed predominately in the liver (hepatic) and to a lesser extent in the gastrointestinal tract (extra-hepatic) were of interest. HAAs are rapidly absorbed from the gastrointestinal tract and bioactivation and/or detoxification may occur either locally or in the liver; thus, expression in these sites was hypothesized to most likely contribute to the formation of DNA adducts in blood leukocytes (17,18).

i. Bioactivation via Cytochrome P450

CYP1A2 is largely responsible for the hepatic bioactivation of HAAs into *N*-hydroxy HAA species whereas extrahepatic tissue (colorectum) metabolism is supported by CYP1A1 and CYP1B1 (14). Therefore, the activity of CYP1A2 and to a lesser extent CYP1A1 and 1B1 has the potential to modify the effect of HAAs on DNA adduct formation. Individuals who carry CYP1A2/1A1/1B1 rapid phenotype are thought to be at increased risk of cancer due to an enhanced ability to bioactivate carcinogens such as HAAs which can subsequently increase the formation of HAA-DNA adducts. CYP1A1*2A, CYP1A2*1B, CYP1A2*1D, CYP1A2*1F and CYP1B1*2, CYP1B1*3 and CYP1B1*4 were identified polymorphisms of relevance with an

MAF of greater than 10%; ranked according to priority, CYP1A1*2A, CYP1A2*1F and CYP1B1*2 were genotyped in Phase1.

ii. Bioactivation by *N*-acetyltransferases

NATs transform the *N*-hydroxy-HAA species to reactive esters that attack DNA (17). NAT1 is primarily expressed in extrahepatic tissues like colorectal tissues and NAT2 is expressed predominately in the liver but also in the intestinal epithelium. Three NAT- associated acetylation phenotypes based on polymorphisms have been described; fast, intermediate and slow (39). NAT1 and NAT2 fast acetylators have a greater ability to conjugate HAAs into highly unstable esters that form adducts with DNA; therefore polymorphisms in NAT1 and NAT2 can potentially increase the formation of DNA adducts. Ranked according to priority, the alleles NAT2*5K and NAT1*10 are commonly utilized in epidemiologic studies to determine fast, intermediate versus slow acetylators and were genotyped in Phase 1.

iii. Bioactivation by Sulfotransferase

The human SULTs catalyze the sulfation of *N*-hydroxy HAAs and thus, like NATs, SULTs enhance the mutagenicity of HAAs particularly for *N*-hydroxy derivatives of PhIP. SULT1A1 is the most widely studied polymorphic SULT with wide tissue distribution (40). Individuals who carry higher SULT1A1 enzymatic activity likely have a higher activity to conjugate HAAs into reactive metabolites, hypothesized to be associated with an increase in the formation of DNA adducts. SULT1A1*2 is the only polymorphism with a MAF of greater than 10%; however ranked according to priority it was not genotyped in Phase 1.

iv. Detoxification by Glutathione S-Transferase

GSTs are an important class of enzymes involved in the detoxification of the carcinogenic intermediates conjugated by NATs (*N*-acetoxy-HAA). Only *N*-acetyoxy-PhIP has

been reported to be a substrate for GSTs, in which GSTs diminished the binding of this reactive PhIP metabolite to DNA. Human GSTA1-1 was reported to most efficiently detoxify this carcinogen. GSTA1*B was the only set of polymorphisms within GSTA1 that had a MAF of greater than 10% and was selected for genotyping in Phase 1.

v. Detoxification by UDP-glucuronsyltransferase

UGTs catalyze the detoxification of HAAs through glucuronidation and elimination of HAAs. The human *UGT1A* family of enzymes is principally involved in the *N*-glucuronidation of PhIP and most likely MeIQx as well (19). Evidence shows that the human UGT1A1 isoform is the most active enzyme involved in N-glucuronidation of PhIP and N-hydroxy-PhIP (19). UGT1A1*28 was identified as the only set of polymorphisms with a MAF greater than 10%; however ranked according to priority it was not genotyped in Phase 1(20).

3.4 Phase 1: Repair of DNA Adducts by Nucleotide Excision Repair and Base Excision Repair

For Phase 1, genes involved in the repair of DNA adducts (i.e. the removal of DNA adducts) were of interest as higher enzymatic activity to repair DNA adducts was hypothesized to decrease the steady-state levels of bulky DNA adducts in blood leukocytes.

Nucleotide excision repair (NER) is thought to be the most relevant DNA repair pathway to the repair of DNA adducts (6). The NER pathway removes lesions, such as DNA adducts, that cause distortion to the DNA helix. The general schema of DNA repair by NER follows that once the site of damage is recognized, DNA is unwound around the lesion (e.g. DNA adduct) by helicase enzymes. Unwinding creates areas in the double stranded DNA strand that are single stranded; the damaged strand of DNA is then incised at these single-stranded areas and the remaining gap is then filled by DNA polymerase, an enzyme involved in DNA synthesis (21).

Though NER is thought to be the predominant pathway involved in the repair of DNA adducts, components of base excision repair (BER) have also been reported to be involved in the repair of DNA adducts (7,9,10). BER is one of the most active DNA repair processes that allows the specific recognition and excision of a damaged DNA base (22). The majority of damage processed by the BER pathway is generated by the attack of reactive oxygen species, which are products of normal cellular metabolism. BER can be divided into three stages: damage recognition, removal of the damaged base and filling the remaining gap by DNA polymerase (21).

3.4.1 Phase 1: Polymorphisms in Genes Involved in Repair of DNA Adducts

Only seven studies have investigated the relationship between polymorphisms in DNA repair genes and bulky DNA adduct levels. The genes and associated polymorphisms that were considered for this thesis included genes involved in NER (XPA rs1800975, XPC rs2228001, XPD rs13181, XPD rs1799793 and ERCC1 rs11615) and BER (XRCC1 rs25487). Associations between polymorphisms in XPA, XPD and XRCC1, and bulky DNA adducts have been reported and were thus prioritized in Table 3.5.

i. Xeroderma pigmentosum group A

The XPA protein is required for NER and is involved in the DNA damage recognition process. XPA forms a complex with another protein and preferentially binds to damaged DNA; this complex has been implicated as a key component in the earliest stage of damage recognition. Recent evidence has found that the binding of this complex to damaged DNA can dramatically increase the repair rate of certain DNA adducts (23); one epidemiologic study has previously reported on XPA –4G>A as a genetic determinant of bulky DNA adducts (6). The XPA –4G>A polymorphism has a MAF of greater than 10% and was genotyped in Phase 1.

ii. Xeroderma pigmentosum group D

The XPD protein plays a role in the NER pathway. XPD works as a helicase enzyme used to separate the double helix. Among other downstream effects, mutations in the *XPD* gene can give rise to repair defects. A number of polymorphisms in the *XPD* gene have been reported, two of which (XPD 934G>A and 2251A>C) have been investigated in relation to bulky DNA adducts with evidence of an association (7,8,11). Both polymorphisms in *XPD* have a MAF of greater than 10% and were genotyped in Phase 1.

iii. X-ray repair cross complementing protein group 1

XRCC1 is a DNA repair gene whose protein product is involved in a number of repair pathways beyond BER and therefore has been extensively studied (20). The protein encoded by *XRCC1* is essential to the repair of single strand breaks and to BER; XRCC1 is thought to act as a scaffold protein (i.e. a protein that interacts and/or binds with multiple members within a pathway, tethering them into a complex) and is involved in numerous repair pathways. Greater than 60 SNPs have been identified in the *XRCC1* gene; 1196A>G is most relevant to Caucasian populations. Two studies have reported an association between XRCC1 1196A>G and bulky DNA adduct levels (9,10). The XRCC1 1196A>G polymorphism has a MAF of greater than 10% and was genotyped in Phase 1.

3.5 Summary of Polymorphisms of Interest to Phase 1

In summary, the polymorphisms of highest priority to Phase 1 along with their demonstrated functional effects are listed in Table 3.6. It was hypothesized that higher bioactivation and/or lower detoxification and/or DNA repair activity would confer susceptibility to dietary HAA intakes and increase bulky DNA adduct levels.

Table 3.6. Genetic factors of interest to Phase 1 and the functional effects of each allele

Gene	rs[1]	Alleles	Functional Effect	References
CYP1A1*2A	4646903	T	Higher bioactivation activity	(24)
		C	Referent	
CYP1A2*1F	762551	A	Higher bioactivation activity	(25,26)
		C	Referent	
CYP1B1*2	10012	G	Higher bioactivation activity	(3)
		C	Referent	
	1056827	T	Higher bioactivation activity	(3)
		G	Referent	
NAT1*10	1057126; 15561	1+	Rapid/Intermediate	(27)
		0	Slow	
NAT2*5K	1041983; 1801280	0/1	Rapid/Intermediate	(28,29)
		2+	Slow	
GSTA1*B	3957356	*B	Lower detoxification activity	(30)
		*A	Referent	
XPA	1800975	C	Lower DNA repair activity	(6,23)
		T	Referent	
XPD	13181	C	Lower DNA repair activity	(6,20)
		A	Referent	
XPD	1799793	A	Lower DNA repair activity	(8)
		G	Referent	
XRCC1	25487	A	Lower DNA repair activity	(7,20)
		G	Referent	

[1]Reference SNP identification number

3.6 Phase 2: Dietary HAA Intake and Risk of Colorectal Adenomas

In a cross-sectional study of 342 individuals undergoing a screening colonoscopy, Phase 2 investigated the relationships between: a) exposure to dietary HAAs and colorectal adenoma prevalence and; b) exposure to meat mutagenicity on the risk of colorectal adenoma. Of interest to Phase 2 are the selected polymorphisms of Phase 1 as well as polymorphisms in genetic factors related to the repair of DNA damage (e.g. mutations) induced by HAAs/DNA adducts (Figure 3.3). The following sections present the gene selection strategy utilized for Phase 2 to identify and prioritize polymorphisms in genes involved in the repair of DNA damage induced by HAAs.

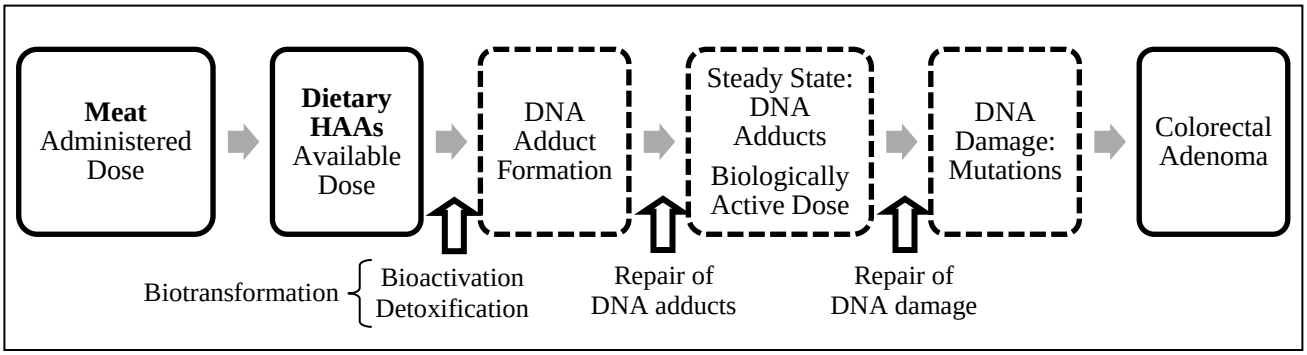

Figure 3.3: Conceptual model of Phase 2; genes of interest include those involved in the biotransformation of HAAs and the repair of DNA adducts and damage induced by DNA adducts. Solid lines indicate measured variables of interest in Phase 2.

3.6.1 DNA Adducts, Mutations and Cancer

HAAs, like the majority of chemical carcinogens, form DNA adducts. Mutagenesis is one pathway that links DNA adduct formation and cancer. In general, mutational frequency has been reported to increase linearly with DNA adduct levels (31); though it has been demonstrated that different DNA adducts have varying capabilities of inducing mutations (32).

3.6.2 DNA Damage Assays

As a starting point, to identify DNA repair genes relevant to the repair of damage induced by HAAs, DNA damage assays which have demonstrated associations with HAAs were first identified. The effect of PhIP and MeIQx on markers of DNA damage which includes micronucleus formation, DNA strand breaks and sister chromatid exchanges have been demonstrated (33-36). Less is known of the effects of DiMeIQx on markers of DNA damage. These DNA damage assays represent different forms of DNA damage and have all been associated with cancer risk in human populations.

i. Micronucleus Formation

The presence of a micronucleus has been used as a surrogate biomarker of chromosome breakage or loss, genetic instability and/or exposure to environmental mutagens or carcinogens

(37). The occurrence of a micronucleus in blood lymphocytes has for many years been applied as a biomarker of genotoxic exposure (37).

ii. DNA Strand Breaks

Both single and double stranded DNA breaks are detrimental to the cell; a break in DNA encoding a key gene can cause cell death. DNA strand breaks can be quantified in a semiquantitative manner using the comet assay; this assay has been used to analyze damage and rate of repair in epidemiologic studies (38).

iii. Sister Chromatid Exchanges

Less is known about the molecular basis of sister chromatid exchanges, but sister chromatid exchanges appear to be the consequence of DNA replication on a damaged template (39). The readily quantifiable nature of sister chromatid exchanges and the demonstrated ability of genotoxic chemicals to induce a significant increase in sister chromatid exchanges have resulted in this end point being used as a biomarker of exposure to genotoxic agents in human populations (39).

3.6.3 Phase 2: DNA Repair Polymorphism Selection Strategy

Literature searches were conducted to identify associations between polymorphisms in DNA repair genes and micronucleus formation, DNA strand breaks and sister chromatid exchanges. Candidate polymorphisms in specific genes were only considered if their MAF was greater than 10%. The DNA repair pathways, genes and polymorphisms with demonstrated associations with micronucleus formation, DNA strand breaks and sister chromatid exchanges in disease-free populations are outlined in Table 3.7. All associations between candidate polymorphisms and these assays were modest and thus, the magnitude of effects was not used for prioritization.

Prioritization was conducted based on the identification of: 1) epidemiologic evidence supporting an interaction between the candidate polymorphism and HAA exposure/meat consumption in relation to CRC or colorectal adenoma risk and; 2) epidemiologic evidence supporting an association between the candidate polymorphism and CRC or colorectal adenoma risk. Figure 3.4 outlines a schematic illustrating the gene selection strategy employed in Phase 2. Table 3.8 is a summary table that prioritizes the polymorphisms of interest to Phase 2 using the lines of evidence described.

Table 3.7. DNA repair pathways, and genes and polymorphisms of interest for Phase 2 with a MAF greater than 10% and demonstrated association with micronucleus formation, DNA strand breaks and sister chromatid exchanges.

Phenotypic Assay	DNA Repair Pathway	Genes	rs[1]
Micronucleus formation	Base Excision Repair	ADPRT	1136410
	Base Excision Repair	XRCC1	25487
	Nucleotide Excision Repair	ERCC1	11615
	Nucleotide Excision Repair	ERCC6	2228526
	Nucleotide Excision Repair	XPD	13181
	Homologous Repair	XRCC3	861539
	Cell Cycle Checkpoint	P53	1625895
	Cell Cycle Checkpoint	ATM	600931
	Cell Cycle Checkpoint	ATM	189037
	Cell Cycle Checkpoint	ATM	624366
	Cell Cycle Checkpoint	ATM	227092
DNA strand breaks	Base Excision Repair	XRCC1	25487
	Base Excision Repair	APE1	1130409
	Nucleotide Excision Repair	XPD	13181
	Nucleotide Excision Repair	XPC	2228001
	Nucleotide Excision Repair	XPD	1799793
	Nucleotide Excision Repair	XPC	2228000
	Nucleotide Excision Repair	XPA	1800975
	Homologous Repair	NBS1	1805794
	Homologous Repair	XRCC3	861539
	Cell Cycle Checkpoint	ATM	228589
	Cell Cycle Checkpoint	ATM	652311
Sister chromatid exchanges	Base Excision Repair	XRCC1	25487

[1]Reference SNP identification number

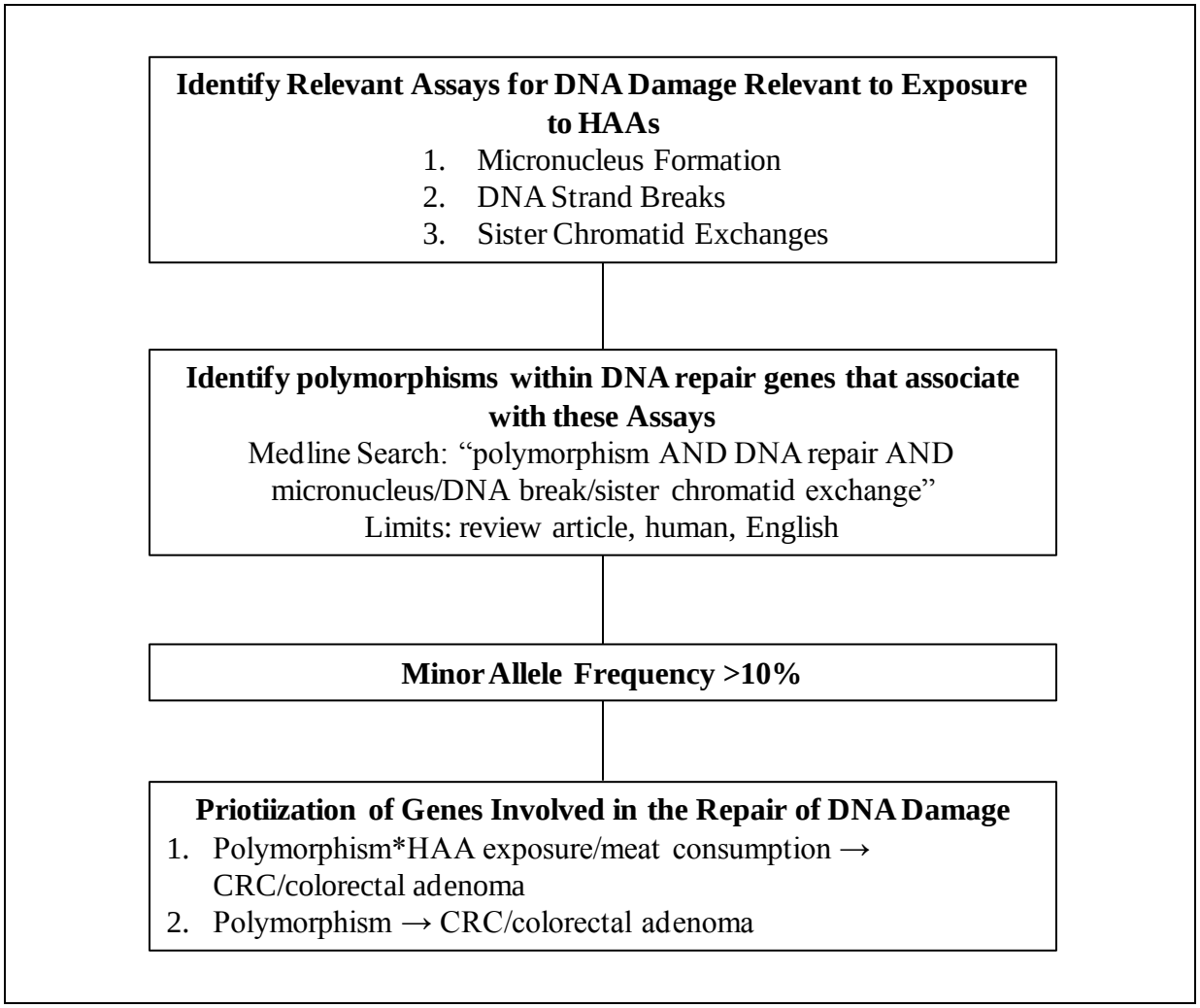

Figure 3.4: Schematic of Phase 2 gene selection strategy

Table 3.8. Summary table of polymorphisms of interest to Phase 2. Unshaded rows indicate the polymorphisms genotyped in Phase 2.

Gene	Polymorphism	rs[1]	MAF	Evidence of Interaction with HAAs/Meat on CRC[2]	Evidence of Effect on CRC[2]
ADPRT	2285T>C	1136410	11%	Yes	No
XRCC1	1196A>G	25487	47%	Yes/No	Yes/No
XPD	2251A>C	13181	27%	Yes/No	Yes/No
XPD	934G>A	1799793	41%	Yes/No	Yes/No
XRCC3	722C>T	861539	45%	Yes	Yes/No
XPC	2815C>A	2228001	34%	Yes	No
ERCC1	354T>C	11615	36%	N/A	Yes/No
ERCC6	3289A>G	2228526	20%	N/A	Yes/No
p53	672+62A>G	1625895	27%	N/A	No
ATM	922-356C>T	600931	47%	N/A	N/A
ATM	-111G>A	189037	34%	N/A	N/A
ATM	3746+491G>C	624366	47%	N/A	N/A
ATM	*548G>T	227092	49%	N/A	N/A
ATM	-736A>T	228589	33%	N/A	N/A
ATM	*3834G>A	652311	36%	N/A	N/A
APE1	444T>G	1130409	43%	N/A	Yes/No
XPC	1385C>T	2228000	36%	N/A	No
XPA	-4G>A	1800975	40%	No	Yes
NBS1	553G>C	1805794	31%	N/A	No

[1]Reference SNP identification number
[2] N/A: No study identified; Yes/No: Conflicting evidence

3.7 Overview of the DNA Damage Response

The DNA damage response is a comprehensive and complex set of responses which safeguards the genomic integrity of cells (40). DNA repair mechanisms lay the foundation of DNA damage response which encompasses a sophisticated array of cellular initiatives set in motion as cells are exposed to DNA damaging events (40). Added layers of monitoring such as the activation of cell cycle checkpoints or apoptosis together with DNA repair determine the cellular fate of promoting either cell survival or death (40).

3.7.1 DNA Repair Mechanisms

DNA repair is arguably the most important component of DNA damage response (40). Five main kinds of DNA repair system exist in mammals with overlapping pathways including: 1) direct reversal; 2) base excision repair (BER); 3) nucleotide excision repair (NER); 4) homologous recombination and non-homologous end joining and; 5) mismatch repair (21). Collectively, these pathways are orchestrated by more than 150 proteins, which enable the response to a wide array of DNA-damaging events. Associations between micronucleus formation, DNA strand breaks and sister chromatid exchanges, and components of BER, NER and homologous recombination have been reported.

i. Base-Excision Repair

The BER pathway was discussed in detail in Section 3.4 in the context of Phase 1 (i.e. the effect of variation in BER activity on determining DNA adduct levels). In the context of Phase 2, variations in BER activity have been associated with micronucleus formation, DNA strand breaks and sister chromatid exchanges. The BER pathway is one of the most active DNA repair processes and is involved in the repair of DNA damage formed from oxidation, spontaneous base hydrolysis or modification by alkylating agents.

ii. Nucleotide Excision Repair

The NER pathway was discussed in detail in Section 3.4 in the context of Phase 1 (i.e. the effect of variation in NER activity on DNA adducts). In the context of Phase 2, variations in NER activity have been associated with micronucleus formation and DNA strand breaks. The NER pathway is a flexible damage-repair mechanism that recognizes larger helix-distorting lesions that occur by chemical modifications of DNA bases including exposure to environmental mutagens like HAAs, UV light, tobacco smoke and reactive oxygen species (38). NER has the ability to eliminate a wide diversity of structurally unrelated DNA lesions since it recognizes damage regions of DNA by their abnormal structure (22). Beyond the repair of bulky adducts induced by chemicals like HAAs, NER has also been shown to be involved in the removal of minor base damaged induced by alkylating and oxidizing agents that are not helix distorting. As well, NER has been demonstrated to help remove oxidatively generated DNA lesions.

iii. Homologous Recombination

Homologous recombination has been associated with micronucleus formation and DNA strand breaks. Homologous recombination is a multistep process that is responsible for the repair of double strand breaks which uses the genetic information on a duplicate DNA strand for repair of the damaged bases (21).

3.7.2 Cell Cycle Checkpoints

To prevent cells with damaged DNA from undergoing replication, genes involved in cell-cycle checkpoints respond to DNA damage by promoting cell cycle arrest (40). This arrest allows more time for the repair enzymes to repair DNA prior to DNA synthesis (40). Likewise, to avoid the proliferation of damaged DNA, cells may also undergo apoptosis. Apoptosis is considered the last resort mode for cells if repair of DNA damage is slow or incomplete (40).

Cells appear to possess an internal "apoptotic clock" which is set to activate the induction of apoptosis when the time runs out (40). Associations between micronucleus formation, DNA strand break and sister chromatid exchanges, and genes involved in cell cycle checkpoints have been reported; however ranked according to priority, none of the genes involved in cell cycle checkpoints were genotyped in Phase 2.

3.7.3 Phase 2: Polymorphisms in Genes Involved in the Repair of Damage Induced by HAAs

The following subsections are organized according to the types of DNA repair pathways (i.e. BER, NER and homologous recombination) involved in the repair of DNA damage induced by exposure to HAAs (i.e. as demonstrated by their association with micronucleus formation, DNA strand breask and sister chromatid exchanges). Within each subsection a brief outline of the relevant genes and associated polymorphisms in the repair mechanism is described. Recall that Table 3.8 provides a summary that prioritizes the polymorphisms of interest to Phase 2 using the lines of evidence described in Section 3.6.4. Only polymorphisms that were genotyped in Phase 2 are presented in the following subsections.

3.7.4 Phase 2: Polymorphisms in Genes Involved in Base Excision Repair

i. X-ray repair cross-complementing group 1

XRCC1 encodes a scaffold protein to facilitate the BER processes (41). The XRCC1 1196A>G polymorphism has been studied extensively in relation to mutagen sensitivity and cancer risk. Specifically, associations with DNA strand breaks and sister chromatid exchanges have been reported (42,43). Two studies have investigated the interaction between XRCC1 1196A>G and meat consumption in relation to CRC risk (41,44); one of which reported a

significant interaction (44). Seven studies have investigated the association between XRCC1 1196A>G and CRC risk (41,44-49); four report an association (41,45,47,50).

ii. ADP-ribosyltransferase:

ADP-ribosyltransferase (ADPRT) interacts with the scaffold protein XRCC1 in a complex to facilitate BER (41). An association between ADPRT1 2285T>G polymorphism and micronucleus formation has been reported (51). One study by Brevik et al (44) has reported an interaction between ADPRT1 2285T>G and meat consumption on CRC risk. However, there is no evidence of a main effect of ADPRT1 2285T>G on CRC risk (44,52).

3.7.5 Phase 2: Polymorphisms in Genes Involved in Nucleotide Excision Repair

i. Xeroderma pigmentosum group D

Among the known genetic polymorphisms in DNA repair genes, polymorphisms in *XPD* have been the most studied cancer susceptibility genes (41). *XPD* encodes a helicase enzyme that participates in NER in the unwinding of the helix in the region of damaged DNA (41). The XPD polymorphisms 934G>A and XPD 2251A>C have been associated with increased DNA strand breaks (53,54). Two studies have investigated the interaction between the XPD 934G>A polymorphism and dietary intakes of meat in relationship to CRC risk (55,56), one of which reported a significant interaction (55). Likewise, two studies have investigated the interaction between meat consumption and XPD 2251A>C in relation to CRC risk (41,55); Joshi et al (55), reported a significant interaction. There is epidemiologic evidence to support the associations between XPD 934G>A and 2251A>C, and CRC risk. Three (56-58) of nine studies have reported a relationship between XPD 934G>A and CRC risk (41,49,55-61); one (58) of seven studies report a relationship between XPD 2251A>C and CRC risk (48,49,55,56,58-60).

ii. Xeroderma pigmentosum group C

XPC encodes an enzyme that is involved in the damage recognition-complex of NER. One of the most common XPC polymorphisms, XPC 2851C>A, has been reported to affect micronucleus formation (51,62). Hansen et al (56) reported interactions between XPC 2851C>A and meat consumption on CRC risk. However, no study has reported an association between XPC 2851C>A and CRC risk (56,59).

3.7.6 Phase 2: Polymorphisms in Genes Involved in Homologous Recombination

i. X-ray repair cross-complementing group 3

X-ray repair cross-complementing group 3 (XRCC3) participates in homologous recombination to maintain chromosomal stability and repair DNA damage (41). The XRCC3 722C>T polymorphism has been shown to affect micronucleus formation and DNA strand breaks (63,64). Accordingly, one study that investigated the interaction between XRCC3 722C>T and meat consumption found a significant interaction for CRC risk (41). Two (45,61) of seven studies that investigated the effect of XRCC3 722C>T on CRC risk reported a statisitically significant association (45,47-49,57,61,65).

3.8 Summary of Polymorphisms of Interest to Phase 2

In summary, the polymorphisms of highest priority to Phase 2 along with their demonstrated functional effects are listed in Table 3.9. It was hypothesized that higher bioactivation and/or lower detoxification and/or DNA repair activity would confer susceptibility to HAA exposures and increase the risk of colorectal adenomas.

Table 3.9. Genetic factors of interest to Phase 2 and the functional effects of each allele

Gene	rs[1]	Alleles	Functional Effect	References
CYP1A2*1F	762551	A	Higher bioactivation activity	(25,26)
		C	Referent	
CYP1B1*2	10012	G	Higher bioactivation activity	(3)
		C	Referent	
	1056827	T	Higher bioactivation activity	(3)
		G	Referent	
NAT2	1041983	C	Rapid/Intermediate	(28,29)
		T	Slow	
GSTA1*B	3957356	*B	Lower detoxification activity	(30)
		*A	Referent	
XPA	1800975	C	Lower DNA repair activity	(6,23)
		T	Referent	
XPD	13181	C	Lower DNA repair activity	(6,20)
		A	Referent	
XPD	1799793	A	Lower DNA repair activity	(8)
		G	Referent	
XRCC1	25487	A	Lower DNA repair activity	(7,20)
		G	Referent	
ADPRT	1136410	C	Lower DNA repair activity	(44,66)
		T	Referent	
XPC	2228001	C	Lower DNA repair activity	(56)
		A	Referent	
XRCC3	861539	T	Lower DNA repair activity	(41)
		C	Referent	

[1]Reference SNP identification number

3.9 Conclusions and Perspectives

In summary, this review highlighted the numerous genes involved in the biotransformation of HAAs, the repair of DNA adducts and the repair of DNA damage induced by HAAs. The purpose of this review was to clearly denote the gene selection strategy that was used to prioritize the polymorphisms of interest for Phase 1 and 2 of this dissertation. The strategies were developed separately for Phase 1 and 2 and prioritization was necessary since sample size coupled with allele frequency may limit the power of a study to investigate gene-environment interactions. In addition, the purpose of gene selection was to identify genes of interest that were most likely to modify the relationships of interest to Phase 1 and 2 and not to

identify novel variants of interest. Thus, utilizing established and/or previously demonstrated associations as the basis for selecting genes of interest was another means for prioritization. It was recognized during the review that differences in the distribution of gender, age and ethnicity could have contributed to some of the dissimilar results observed in the literature. Most of the studies assessed individual polymorphisms in genes in relation to the risk of CRC and reported modest effects. Since cancer is a complex multigenic and multistage disease involving the interplay of many genetic and environmental factors, it is unlikely that a single genetic polymorphism within low penetrance genes would have a dramatic effect on cancer. However, the investigation of the combined effects of polymorphisms and environmental factors like HAAs may further clarify the underlying meat-CRC relationship.

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Chapter 4: Phase 1 Study Design and Methods

This doctoral dissertation investigated the relationship between heterocyclic aromatic amines (HAAs) and colorectal adenoma risk in two epidemiologic studies (Phase 1 and 2). Phase 1 investigated the relationship between dietary exposure to HAAs and the levels of potentially carcinogenic bulky DNA adducts in blood leukocytes in a cross-sectional study of 125 healthy volunteers. Phase 1 also evaluated whether the relationship between dietary HAA intake and bulky DNA adduct levels in blood leukocytes is modified by variation in genes involved in the biotransformation of HAAs, and the repair of DNA adducts. This Chapter presents an overview of the study design and methods of Phase 1.

4.1 Phase 1: Dietary HAA Intake and Bulky DNA Adducts

Phase 1 of this research, which was funded by Cancer Care Ontario, was designed to investigate the relationship between dietary exposure to HAAs and levels of potentially carcinogenic bulky DNA adducts in blood leukocytes in a cross-sectional study of 125 volunteers aged 25 to 60 recruited from Queen's University and its surrounding institutions. Data collection consisted of: 1) a meat consumption module that assessed average HAA intake during the previous year; 2) a 12-hour fasting blood sample for quantification of bulky DNA adduct levels and determination of genetic susceptibility and; 3) a questionnaire that collected information on personal characteristics and lifestyle factors that may influence the absorbed doses of dietary HAAs.

To estimate average intake of dietary HAAs, the meat consumption module was linked with the National Institutes of Health Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease (CHARRED) mutagen database which is a software application that details the specific concentrations of HAAs in a variety of cooked meats (1). Bulky DNA

adducts levels in white blood cells were determined by ^{32}P-postlabelling (2). Common polymorphisms in genes involved in the biotransformation of HAAs and DNA repair were also genotyped in DNA isolated from blood to determine effect modification by genetic susceptibility on the HAA-DNA adduct relationship.

4.1.1 Study Subjects

The study population included 125 male and female volunteers ages 25 to 60, who self-reported to be generally healthy. This age range was selected based on the premise that it would include a population with stable dietary habits and would represent the relevant exposure period for cancer development. Individuals, who were vegetarians and thus not exposed to HAAs, were excluded. People previously diagnosed with colorectal cancer, colorectal adenoma, Crohn's disease, inflammatory bowel disease or colitis were not eligible for this study.

4.1.2 Recruitment

Participants were recruited within Queen's University and its surrounding institutions. Advertisements on public bulletin boards within the University and other institutions described the study and invited potential participants to contact the study coordinator. Efforts were made during recruitment to obtain approximate equal representations in 10-year age groups. Those responding were screened for eligibility and an appointment was scheduled at the study recruitment lab, Carruthers Hall, Queen's University, Kingston, Ontario. Participants were instructed to abstain from food and drink for twelve-hours prior to the clinic visit. At the morning of the clinic visit, the study coordinators met with the participant in order to measure height and weight, administer the meat consumption module and questionnaire, and obtain a blood sample.

4.1.3 Data Collection

Sources of information from each participant came from a meat consumption module, questionnaire and blood sample.

i. Meat Consumption Module

Participants completed a meat consumption module that estimated the average consumption of dietary HAAs. Dietary HAA exposure was estimated by obtaining the average frequency of consumption, usual level of doneness and usual serving size of nine commonly consumed meats during summer (April to October) and winter (November to March) months (Appendix 2). Meat items considered consist of those that have high HAA content and included: hamburger, beef steak, fried chicken, chicken eaten with skin and without skin, pork chop, ham slice, sausage and bacon.

ii. Questionnaire

Height and weight of each participant was measured by the study coordinators and participants completed a study questionnaire that collected information on personal characteristics and lifestyle factors. Relevant covariates included sex, age, ethnicity, smoking, fruit and vegetable consumption and alcohol consumption (Appendix 2).

iii. Blood Sample

Fasting blood samples were collected by a trained phlebotomist. Twenty-four milliliters of fasting blood was collected in the morning between 8 to 10 am. The blood specimen was drawn into two-10 ml and one-4 ml ethylenediaminetetraacetic acid-containing tubes and centrifuged within half an hour of collection to obtain buffy coat (leukocytes) for storage at -20˚C.

4.1.4 Exposure Assessment – Dietary Heterocyclic Aromatic Amines

i. Development of the Meat Consumption Module

The development of the meat consumption module for Phase 1 utilized the meat items list from the CHARRED mutagen database as the starting point for questionnaire development. The CHARRED mutagen database details the specific concentrations of PhIP, MeIQx, DiMeIQx and other carcinogens in commonly consumed meat items according to the specific types of cooking method and preferred doneness level. Existing questionnaires that have previously been linked to the CHARRED mutagen database including the CHARRED sample questionnaire and the Arizona Meat Preparation Questionnaire were adapted for use in Phase 1 by verifying whether the meat items assessed included commonly consumed foods of Canadians. This was performed by comparing both the CHARRED sample questionnaire and the Arizona Meat Preparation Questionnaire with the Canadian version of the Diet Habits Questionnaire (Canadian version) (3). After a review of the literature on the formation of dietary HAAs, doneness level and serving size were ascertained as important determinants of dietary HAA formation. Therefore, coloured photographs depicting doneness level of meat items and serving sizes were included with the meat consumption module to standardize the assessment of these domains (Sinha, R.; personal communication). During the development of the meat consumption module, the stability of dietary habits was considered since the measurement of steady-state concentrations of bulky DNA adducts was conceptualized to result from habitual dietary HAA exposure in the past year. Therefore, the meat consumption module captured dietary intakes of HAA separately for summer and winter months. Pilot testing of the meat consumption module among 20 subjects was conducted to ensure clarity of the questions.

ii. Derivation of Dietary Heterocyclic Aromatic Amine Exposures

The average intake of PhIP, MeIQx and DiMeIQx was estimated by linking responses from the meat consumption module to the CHARRED mutagen database. Specifically, the CHARRED mutagen database details specific PhIP, MeIQx and DiMeIQx content (expressed in nanograms per gram of meat (ng/g)) in 120 categories representing the composite of different meat items, cooking methods and doneness levels. The meat consumption module collected information on the number of times per day/per week/per month of each meat item consumed according to the type of cooking method, the usual preferred level of doneness, and the usual serving size separately for summer and winter months.

Linking the CHARRED mutagen database to the responses in the meat consumption module, the average intake of dietary PhIP, MeIQx and DiMeIQx was derived by multiplying the specific HAA content by the serving size by the frequency of intake per day. For example, a pan-fried steak cooked to well-done has 6.53 ng of PhIP content per gram of meat. An individual who self-reported to consume a medium serving (112 g) of pan-fried steak cooked to well-done on average three times per week would thus be exposed to 2,194.08 ng of PhIP per week (6.53 ng * 112 g = 731.36 ng of PhIP; 731.36 ng of PhIP * 3 days per week = 2,194.08 ng of PhIP per week) or 313.44 ng of PhIP per day (2,194.08 ng of PhIP per week / 7 days per week = 313.44 ng of PhIP per day). The total PhIP, MeIQx and DiMeIQx intake was summed across the nine meat items separately for summer and winter months; dietary PhIP, MeIQx and DiMeIQx intake in the past year was then averaged across summer and winter months.

4.1.5 Outcome Assessment – Bulky DNA adduct in Blood Leukocytes

DNA from blood leukocytes of 125 participants was isolated using 5'Prime ArchivePure^{TM} DNA Blood Kits; isolation was conducted at Queen's University in the

laboratory of Dr. Massey, Queen's University, Kingston, Ontario. Concentration and purity were determined spectrophotometrically by absorbance at 260 and 280 nm. Determining DNA yield (calculated based on the measured DNA concentration and the known volume of each sample) and ensuring purity (based on the ratio of absorbance at 260/280 nm) was necessary for optimum performance of both DNA adduct quantification and genotyping. Following isolation, 5 µg of DNA was dried and samples were shipped at room temperature to the Laboratory of Drs. Godschalk and van Schooten at the University of Maastricht in the Netherlands to perform the ^{32}P-Postlabelling analysis.

i. ^{32}P-Postlabelling

^{32}P-Postlabelling analysis is a sensitive method for the detection of carcinogen-DNA adducts (4). The procedure involves four main steps: enzymatic digestion of DNA into single nucleotides; enrichment of the carcinogen-adducted nucleotides; radiolabelling of the adducts using ^{32}P and chromatographic separation of labelled adducts for quantification measured by their radioactive decay (4). Bulky DNA adducts were analyzed using the butanol enrichment procedure of the ^{32}P-postlabelling assay (Appendix 3) (5). Out of the 125 participants, 117 had adequate DNA yields (5 µg) for reliable quantification of bulky DNA adducts. For 18 participants, the assay was unsuccessful and thus, for 99 of 117 participants a quantifiable level of bulky DNA adducts was obtained.

ii. Assessment of Reliability/Quality Control

The ^{32}P-postlabelling assay had a detection limit of approximately 1 adducts per 10^9 nucleotides. Due to the limited sample availability and costs, samples were not assessed in replicates. To obtain a measure of reliability, a quality control sample that had a detectable level

of bulky DNA adducts was analyzed in duplicate in every batch; the coefficient of variation of the quality control sample was 17%.

4.1.6 Genetic Assessment – Genes Involved in the Biotransformation of HAAs and the Repair of DNA Adducts

Polymorphisms in genes involved in the biotransformation of HAAs and the repair of DNA adducts were determined utilizing DNA isolated from blood leukocytes at the Queen's Laboratory for Molecular Pathology, Queen's University, Kingston, Ontario. Genetic factors that may modify the relationship between dietary HAA exposure and HAA-DNA adducts included polymorphisms in the *CYP1A1*, *CYP1A2*, *CYP1B1*, *NAT1*, *NAT2*, *GSTA1*, *XPA*, *XPD* and *XRCC1* genes. Selection of these genetic factors was discussed in detail in Chapter 3. Specifically, polymorphisms in *NAT1* (rs1057126, rs15561), *NAT2* (rs1041983, rs1801280), *XPD* (rs13181, rs1799793) and *XRCC1* (rs25487) were genotyped using Applied Biosystems Taqman® SNP Assays. Polymorphisms in *CYP1A1* (rs4646903), *CYP1A2* (rs726551), *CYP1B1* (rs10012, rs1056827), *XPA* (rs1801280) and *XRCC1* (rs25487) were genotyped using polymerase chain reaction-restriction fragment length polymorphism according to previously published methods (6-14). Note that GSTA1*B (rs3957356) was initially selected as a genetic factor of interest to Phase 1; however, due to the inability to replicate the published protocol GSTA1*B was not genotyped (9,15).

4.1.7 Covariate Assessment

Information was collected in the study questionnaire on personal, lifestyle and dietary factors that may determine bulky DNA adducts (age, sex, smoking, environmental tobacco smoke and fruit and vegetable consumption) and induce enzymes that may influence DNA adduct formation (smoking, environmental tobacco smoke and alcohol consumption) (Appendix

2). Weight and height were measured at the time of the interview since an association between body mass index (BMI) and bulky DNA adduct levels has been previously reported. Specific characterizations of potential covariates considered are listed as follows.

i. Characterization of Personal Characteristics

Age and Sex: Age and sex were self-reported by the participants in the questionnaire. Age was considered as both a continuous and categorical variable; categories, based on the sampling strata used, included 25 to 34 years, 35 to 44 years, 45 to 54 years and greater than 55 years.

Ethnicity: Individuals were asked to check White, Chinese, South Asian, Black, Native/Aboriginal, Arab/West Asian, Filipino, South East Asian, Latin American, Japanese, Korean or Other, as the ethnicity for each of their maternal and paternal grandparents. Participants who indicated that each of their maternal and paternal grandparents were White were classified as 'White' and participants who indicated that one or more of their maternal and paternal grandmother and grandfather were one of Black, First Nations, Native or Aboriginal peoples of North America, Chinese, Japanese, Filipino, Korean, South Asian, South East Asian, Arab or West Asian, Latin American or Other were classified as 'Other.'

Body Mass Index (BMI): Weight and height was measured at the time of the interview by the study coordinator. BMI was calculated as the ratio of weight to the square of height (kg/m^2). Categories of BMI were based on the cut points used by the World Health Organization (16). Specially, a BMI of less than 18.50 kg/m^2 was considered 'underweight', between 18.5 and 24.99 kg/m^2 'normal', between 25.00 and 29.99 'overweight' and a BMI of greater than 30.00 was considered 'obese'. The 'underweight' (BM<18.50) category was grouped with 'normal' due to a small cell size (n=2).

ii. Characterization of Lifestyle and Dietary Factors

Smoking: Participants were placed in one of three categories for smoking status: 'never smoker', 'past/ever smoker', or 'current smoker'. 'Never smoker' was defined as those who had not smoked 1 cigarette in the past 30 days and had never smoked at least 1 cigarette a day for 12 months or more. 'Past/ever smoker' was defined as: 1) those who had smoked 1 cigarette in the past 30 days but had not smoked at least 1 cigarette a day for 12 months or more; 2) those who had smoked 1 cigarette in the past 30 days and had smoked at least 1 cigarette a day for 12 months or more but is not currently smoking; 3) those who had not smoked 1 cigarette in the past 30 days but had smoked at least 1 cigarette a day for 12 months or more but is not currently smoking and 4) those who had not smoked 1 cigarette in the past 30 days but had smoked at least 1 cigarette a day for 12 months or more and did not report whether they were currently smoking. 'Current smoker' was defined as those with had smoked at least 1 cigarette in the past 30 days and had smoked at least 1 cigarette a day for 12 months or more and is currently smoking. For regression analysis, 'Current smokers' were grouped with 'Past/ever smoker' due to small cell size (n=5).

Environmental Tobacco Smoke: Exposure to environmental tobacco smoke was dichotomized based on those who self-reported zero or less than one hour of exposure ('non-exposed') and those with greater than 1 hour of exposure ('exposed').

Alcohol Consumption: The average number of specific alcoholic beverages (beer, wine, liquor and coolers/breezers) consumed per week over the past year was abstracted from the questionnaire. This information was used to calculate the average number of drinks per day. Average number of drinks per day was converted to grams of alcohol consumed per day (g/day) using 13.6 grams of alcohol as a standard drink amount (17). Alcohol consumption was

examined as both a continuous and categorical variable; average daily intake of alcohol (g/day) was categorized based on cut points commonly used in the colorectal cancer literature (18). Specifically, alcohol consumption categories included "Abstainers", Light (0-13.6 g/day), Moderate (13.6-54.4 g/day) and Heavy (≥54.4 g/day) (19).

Summary Fruit and Vegetable Consumption: Fruit and vegetable intake was assessed using the Rapid Risk Factor Surveillance System six-item food frequency module; this module has been advocated for quantifying intake of fruit and vegetable consumption (20). The average frequency of consumption of fruit juices, fruits, green salad, potatoes, carrots and other vegetables over the last 12 months expressed in either times per day, times per week or times per month was determined. The frequencies across fruit and vegetable questions were summed and expressed in times per day. Fruit and vegetable intake was considered as both a continuous and categorical variable; categorization was based on quartile representations.

iii. Characterization of Genetic Factors

Phase 1 polymorphisms and their respective genotype categories and functional effects are listed in Table 4.1. Specifically, heterozygotes were grouped with the homozygous variant category to maximize statistical efficiency. In addition, the two polymorphisms in CYP1B1*2, rs10012 and rs1056827, are genetically linked and thus, the haplotype of CYP1B1, combining genotypes of rs10012 'CG or GG' with rs105687 'GT or TT,' was examined in all analyses.

Table 4.1. Genetic factors of interest to Phase 1, their genotype categories and associated functional effects

Function	Gene	rs	Genotypes	Functional Effect	References
Bioactivation	CYP1A1*2A	4646903	TT TC/CC	Higher bioactivation Referent	(21)
	CYP1A2*1F	762551	AA AC/CC	Higher bioactivation Referent	(22,23)
	CYP1B1*2	10012	CG/GG CC	Higher bioactivation Referent	(24)
		1056827	GT/TT GG	Higher bioactivation Referent	(24)
	NAT1[1]	1057126; 15561	1+ 0	Rapid/Intermediate Slow	(25)
	NAT2[2]	1041983; 1801280	0, 1 2+	Rapid/Intermediate Slow	(26,27)
DNA repair	XPA	1800975	CC CT/TT	Lower DNA repair Referent	(28,29)
	XPD	13181	CC/CA AA	Lower DNA repair Referent	(28,30)
	XPD	1799793	AA/AG GG	Lower DNA repair Referent	(12)
	XRCC1	25487	AA/AG GG	Lower DNA repair Referent	(13,30)

[1]NAT1 phenotype is defined by a genotyping NAT1*10. The sum of the variant T1088A and C1095A with null (0) and one or more (1+) variant alleles correspond to the slow and intermediate/rapid genotype.
[2]NAT2 phenotype is defined by a genotyping NAT2*5K. The sum of the variant C282T and T341C alleles with null (0) to one (1) variant alleles corresponding to the rapid and intermediate genotype and with 2 and more variant alleles (2+) corresponding to the slow genotype

4.1.8 Data Validity

i. Genetic Data

For the genetic data, measures were undertaken to assess the intra-assay and inter-assay reliability performed at the Queen's Laboratory for Molecular Pathology. For intra-assay reliability, repeat assays for 10% of the samples were performed for all polymorphisms and concordance was found to be 100%.

In a separate study, participants in Phase 1 were additionally genotyped for all polymorphisms of Phase 1 with the exception of NAT 2 (rs1801280), NAT1 (rs1057126, rs15561) and XPD (rs1799793) using the MassARRAY® iPLEX® Gold – SNP Genotyping assay at Genome Québec, Montréal, Quebec. This overlap in genetic data obtained from two established methods for genotyping allowed for the assessment of inter-assay reliability and concordance was found to be 100%. Further evaluation of genetic data validity was conducted by reviewing published data on genotype distributions of all polymorphisms.

4.1.9 Data Management

All consenting participants were assigned sequential four-digit identification (ID) numbers. This ID number was used to identify participants on all data collection forms and samples. Information abstracted from questionnaires was managed using Microsoft© Access. Genetic data was kept in Excel files and was provided by the Queen's Laboratory for Molecular Pathology and Genome Quebec once data became available. All files containing identifying information such as consent forms were held apart from the data pertaining to the questionnaires, study outcomes and biologic samples in a separate locked filing cabinet only accessible to study personnel.

4.1.10 Data Analysis

Descriptive statistics were generated for all variables considered in this study. The mean, standard deviation (SD), median and 25th and 75th percentiles of all continuous variables were determined, and the frequency of categories was analyzed for categorical variables.

i. Outcome Representation

For the quantification of bulky DNA adducts, 117 out of 125 subjects had adequate DNA yield for the measurement of bulky DNA adducts and for 99 participants the ^{32}P-postlabelling assay was successful in measuring bulky DNA adducts. The normality of bulky DNA adduct levels (expressed as adducts per 10^7 nucleotides) was assessed by graphical analysis (histogram and quantile-quantile plot) and the Shapiro-Wilk test of normality. DNA adduct levels were found to be right-skewed. Therefore, due to deviations from normality and to minimize the influence of outliers on the analysis, bulky DNA adduct measures were logarithmically (log)-transformed.

ii. Exposure Representation

For dietary HAAs, *a priori* three exposure representations were considered: continuous, equal-distant categories and dichotomous. First, the level of dietary HAAs was conceptualized as a continuous variable to increase statistical efficiency. As well, it was hypothesized that the relationship between dietary intakes of HAAs and bulky DNA adduct formation followed a linear relationship and thus, continuous representations would be more representative of the underlying biologic relationship. Categorical representations of dietary HAAs using equal-distant categories were selected as the most appropriate categorical representation since after the examination of the distribution of dietary HAAs no natural cut-points were obvious and quantile representations would yield categories that may not be meaningful (i.e. due to the skew of the

distribution). Specifically, four equal-distant categories were created while ensuring a minimum of 15% of the study population within each category. Using dietary PhIP as an example, the highest category of PhIP was created by categorizing those above the 75th percentile of the study population as Category 4 of PhIP intake (PhIP >248.51 ng/day). Three equal-distant categories were then created by dividing the 75th percentile cut-off (248.51 ng/day) by three yielding Category 1 (0 to ≤82.84 ng/day), Category 2 (82.84 to ≤165.67 ng/day) and Category 3 (165.67 to ≤248.51 ng/day). Finally, dichotomization of dietary HAAs based on the 75th percentile (Category 4) in relation to bulky DNA adducts was also explored; the referent category consisted of those in categories 1, 2 and 3.

iii. Main Analysis

To provide an understanding of the potential for covariates to confound the relationships of interest, bivariate associations between covariates and bulky DNA adducts was examined using least squares regression. Relationships between covariates and dietary HAA intakes (represented using equal-distant categories) were tested using Chi-Square test and Fisher's exact test. In addition, given that the control of confounding requires consideration of the appropriate representation of the covariates of interest (31), variable representations for continuous covariates were selected based on three priorities: 1) parsimonious number of parameters; 2) appropriate representation and 3) interpretable effects. In Phase 1, continuous covariates included age, alcohol consumption and fruit and vegetable consumption. Due to the small sample size of Phase 1, a priority was to model a continuous covariate as continuous where appropriate, to minimize the use of parameters (and degrees of freedom) in the regression model. Appropriate representation of the relationship between each covariate and log-transformed DNA adducts was determined by assessing the linearity of the relationship. Specifically, each

covariate was modeled against log-transformed DNA adducts with the use of a single linear term and the presence of a non-linear association was assessed via the inclusion of quadratic terms. Upon the detection of the potential for non-linear relationships (as indicated by a partial F statistic with p-value of less than 0.10), the use of categorical representations was selected. Categorical representations were selected in place of the use of quadratics to improve the clarity and interpretation of the relationships. It was recognized however that categorical representations will not as efficiently utilize the within-category information as well as the inclusion of polynomials, and therefore potentially be a source of residual confounding. For genetic factors, Chi-Square test was used to evaluate the difference between observed and expected genotype frequencies according to the Hardy-Weinberg Equilibrium; polymorphisms under investigation in Phase 1 are expected to be in Hardy-Weinberg Equilibrium due to the absence of genetic and population factors in affecting allele frequencies.

Correlations between meat-derived carcinogens (PhIP, MeIQx and DiMeIQx) were calculated using Spearman rank correlation. Since dietary HAAs are all estimated from the same dietary measures (frequency of specific meat intake according to different cooking practices, the usual serving size and preferred level of doneness), collinearity was anticipated to be an issue.

Least squares regression was used to investigate the association between dietary HAA intake and bulky DNA adduct levels in separate models, the main analysis included the assessment of the relationship between bulky DNA adduct levels, and specific HAAs (PhIP, MeIQx and DiMeIQx). For both continuous and categorical (equal-distant and dichotomous) representations, the overall p-value derived from the F-statistic was used to determine the statistical significance in all models. For continuous representations, dietary HAAs were expressed per SD and log-linear relationships with bulky DNA adducts were examined;

parameter estimates are thus interpretable as one SD increase in dietary HAAs would result in $(e^{\beta_1} -1)*100$ percent change in bulky DNA adduct levels (per 10^7 nucleotides). For categorical and dichotomous representations, geometric mean DNA adduct levels (i.e. the antilogarithm of the log-transformed mean adduct level) and corresponding 95% confidence interval within categories of exposure were determined.

iv. Assessment of Confounding

Stepwise selection was used to create a parsimonious model of covariates that were predictive of bulky DNA adducts using a liberal p-value of 0.20. A stepwise selection approach evaluates the improvement in fit when each variable is added or dropped via the p-value of the overall F-statistic that describes the variable's contribution to the model. Variables considered included sex, age, ethnicity, alcohol consumption, smoking status, exposure to environmental tobacco smoke, BMI and fruit and vegetable consumption.

v. Effect Modification

Given the available sample size, interactions between exposure to dietary HAAs and polymorphisms in genes involved in the biotransformation of HAAs and DNA repair on determining DNA adduct levels were explored since this research had limited statistical power to elucidate an interaction if one existed. Gene-diet interactions in the main analysis were assessed with the inclusion of cross-products to the multivariable regression models. In the assessment of interactions, dichotomization based on combining categories 3 and 4, and utilizing categories 1 and 2 as the referent was chosen as the exposure representation to maximize statistical power.

4.1.11 Sample Size and Power

Originally, the objective of Phase 1 was to detect and measure specific HAA-DNA adducts with the intention to investigate the associations between dietary HAAs and specific

HAA-DNA adduct levels. However, after extensive method development and validation, the detection of specific HAA-DNA adducts was deemed infeasible. Phase 1 was revised to investigate the relationship between dietary exposure to HAAs and levels of potentially carcinogenic bulky DNA adducts in blood leukocytes.

The relationships between dietary HAA intakes and levels of specific HAA-DNA adducts have not been investigated in human populations. A sample size of 125 was originally selected to provide precision in the estimation of the relationships of interest. A strong relationship was expected to exist between dietary HAAs and specific HAA-DNA adducts in blood. Thus, a small sample size was anticipated to be sufficient to differentiate such a relationship from the null. However, beyond the ability to detect the relationship of interest, an adequate sample size was required to facilitate a degree of precision in estimating the proportion of variance explained (R^2). For multivariable analysis (up to 10 predictors), precision was determined to be ± 0.10 on an R^2 of 0.65 for the dietary HAA and specific HAA-DNA adduct relationship (32). For the relationship between dietary HAAs and the levels of bulky DNA adducts in blood, a lower R^2 was expected since bulky DNA adducts is a more global measure of adducts which incorporates HAA-DNA adducts. Thus, in multivariable analysis (up to 10 predictors), precision was determined to be ± 0.15 on an R^2 of 0.30 for a sample size of 125 (32). When conceptualizing dietary HAA intakes and bulky DNA adduct levels as standardized variables, a slope of ±0.25 is detectable at a significance level of 0.05 and 80% power.

In the analysis of the interactions between genetic factors and dietary HAAs on blood DNA adduct levels, dietary HAA exposures were dichotomized based on combining categories 3 and 4 and using categories 1 and 2 together as the referent category which yielded an approximate equal distribution of study subjects. Power calculations for interaction analyses

were conducted using the statistical software Quanto (33). Calculations were based on: 1) a mean DNA adduct level of 3.3 with a SD of 3.4; 2) a dichotomous representation of at-risk genotypes according to their models of action (as discussed in Section 4.1.7) and; 3) main effects of 1.0 for dietary HAAs and genetic factors (interpretable as having 1 more DNA adduct per 10^7 nucleotides when comparing: a) those with high versus low dietary HAA intake among those without the at-risk genotype or; b) those with the at-risk versus not-at-risk genotype category among those with low dietary HAA intake). For a genotype prevalence of 30%, a study of 125 participants would have greater than 80% power to detect an interaction effect size of 3.5; for a genotype prevalence of 50%, Phase 1 would have greater than 80% power to detect an interaction effect size of 3.0.

4.1.12 Additional Analysis

i. Sex-Specific Analysis

A sex-specific analysis of the relationships between dietary HAA intake and bulky DNA adduct levels in blood leuckocytes was undertaken as an additional analysis due to the emerging hypothesis of an inhibiting effect of estrogens on enzymes involved in the biotransformation in HAAs (34,35). Sex-specific analyses were undertaken with the inclusion of product terms into the multivariable regression model.

4.2 Ethical Considerations

To ensure that this thesis dissertation complied with ethical standards for research on human subjects, Phase 1 was approved by the Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (Appendix 4). With respect to risks and benefits, informed consent and confidentiality, the following should be noted:

Phase 1:

- The risk associated with blood draw was minimal
- As stated in the consent form, there were no direct benefits to the subjects but this research could benefit future studies in understanding the role of HAAs in carcinogenesis
- Throughout the study, procedures were in place related to entry and storage of personal and questionnaire data to protect confidentiality and anonymity at all times. Blood specimens were coded with a non-identifiable subject number assigned at the onset of the study.

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Chapter 5: Phase 1 Results

5.1 Phase 1: Dietary HAA Intake and Bulky DNA Adducts

This doctoral dissertation was designed to inform on the relationship between heterocyclic aromatic amines (HAAs) and colorectal adenoma risk in two epidemiologic studies (Phase 1 and 2). The purpose of Phase 1 was to investigate the relationship between dietary exposure to HAAs and bulky DNA adduct levels in blood leukocytes; the elucidation of this relationship would contribute to the assessment of the potential role of HAAs in carcinogenesis. For the quantification of bulky DNA adducts, 117 out of 125 subjects had adequate DNA yields for the measurement of bulky DNA adducts and 99 of 117 had a quantified level of bulky DNA adducts. Therefore, the results of Phase 1 are limited to the 99 participants with complete data on dietary HAAs and bulky DNA adduct levels. This Chapter presents the results of Phase 1.

5.1.1 Phase 1 Objectives

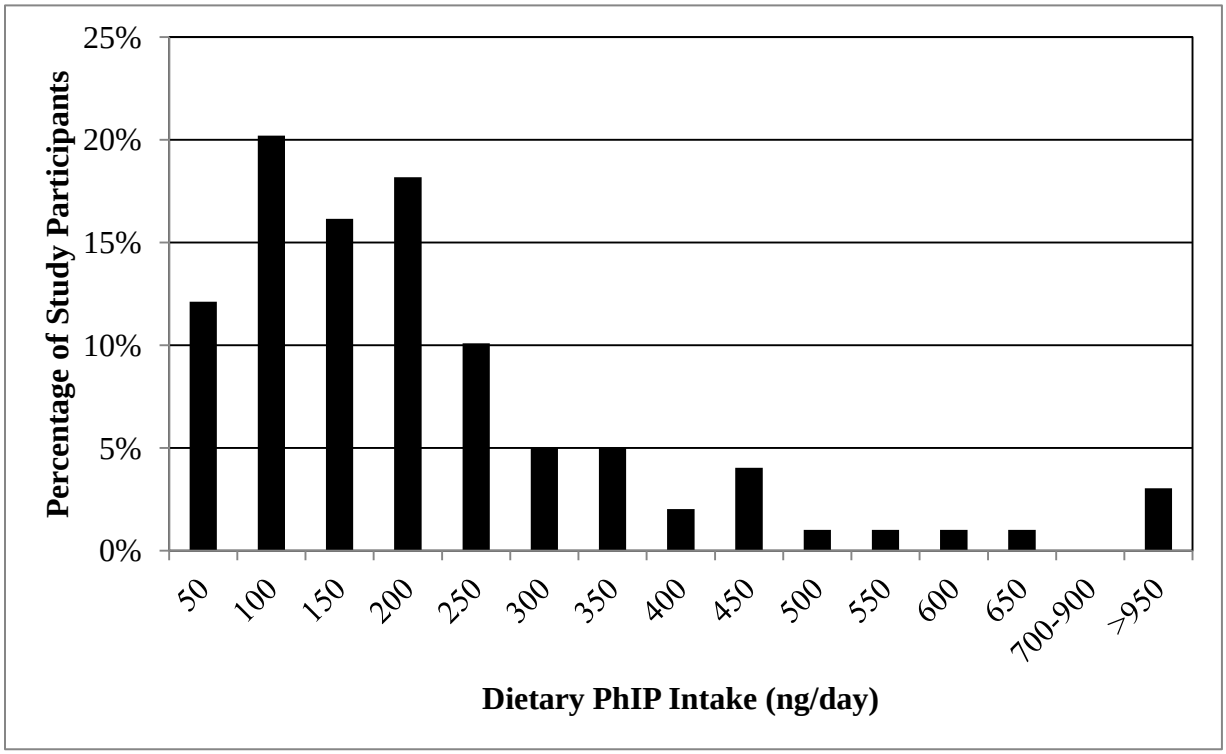
Objective #1 determined the relationship between dietary HAA intake and bulky DNA adduct levels in blood leukocytes. Objective #2 investigated whether the relationship between exposure to dietary HAAs and bulky DNA adduct levels in blood leukocytes was modified by polymorphisms in genes involved in the biotransformation or metabolism of HAAs and/or the repair of DNA adducts.

5.2 Descriptive Statistics

5.2.1 Exposure Distribution - Dietary Heterocyclic Aromatic Amine Intake

The distributions of PhIP, MeIQx and DiMeIQx are presented in Figure 5.1, 5.2 and 5.3 respectively. Table 5.1 presents the summary statistics including mean, standard deviation (SD), median, 25th and 75th percentiles, skewness and W statistic for each exposure of interest among the total study population. The distribution of all exposures of interest is right-skewed. The

Shapiro-Wilk test is a normality test for smaller samples sizes that give a W statistic ranging from 0 to 1, where a value of 1 indicates normality (1). For all exposures of interest, the W



statistic was statistically different from 1 indicating a non-normal distribution ($p<0.05$).

Figure 5.1: Distribution of dietary PhIP in Phase 1 (N=99). Midpoint of each interval is indicated.

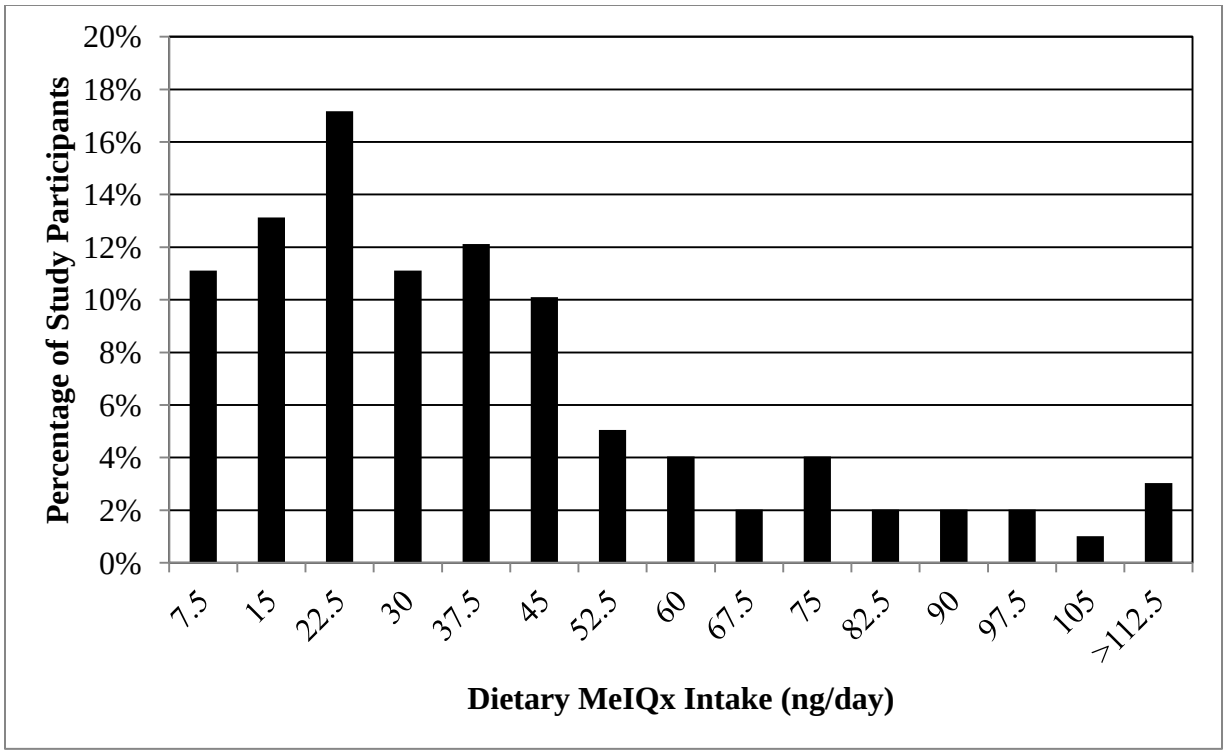


Figure 5.2: Distribution of dietary MeIQx in Phase 1 (N=99). Midpoint of each interval is indicated.

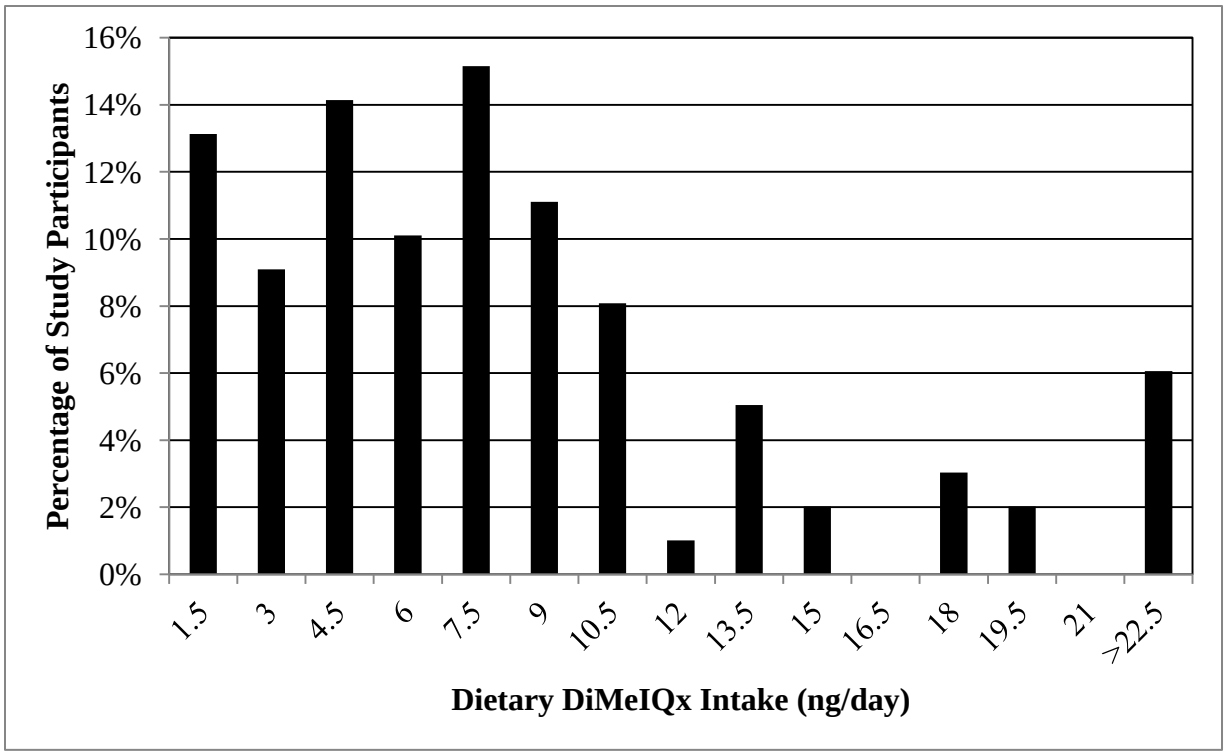


Figure 5.3: Distribution of dietary DiMeIQx in Phase 1 (N=99). Midpoint of each interval is indicated.

Table 5.1. Distribution of dietary PhIP, MeIQx and DiMeIQx in Phase 1 total study population (N=99)

Exposure	Mean	SD	25th Percentile	Median	75th Percentile	Skewness	W
PhIP	202.7	206.3	75.0	154.4	248.5	3.1	0.7
MeIQx	36.2	32.7	15.1	28.8	46.6	2.5	0.8
DiMeIQx	8.3	9.7	3.4	6.2	9.7	3.5	0.7

5.2.2 Outcome Distribution - Bulky DNA Adduct Levels in Blood Leukocytes

Bulky DNA adduct levels ranged from 0.34 to 28.58 adducts per 10^7 nucleotides with a mean and SD of 3.3 and 3.4 adducts per 10^7 nucleotides, respectively. Normality of bulky DNA adduct levels was assessed by graphical (histogram and quantile-quantile (Q-Q) plot) and numerical (Shapiro-Wilk test of normality) univariate analysis. The distribution of bulky DNA adduct concentrations in blood leukocytes of 99 participants is presented in Figure 5.4. The distribution of bulky DNA adducts is right-skewed (Skewness=4.6) and Q-Q plots indicate a deviation from normality (Figure 5.5). The Shapiro-Wilk test W statistic ($W=0.6$; $p<0.01$) was statistically different from 1 indicating a non-normal distribution; thus, bulky DNA adducts were logarithmically (log)-transformed to normalize the distribution of DNA adducts and reduce the influence of extreme observations. Figure 5.5 and 5.6 presents the Q-Q plot of bulky DNA adduct levels before and after log-transformation, respectively. As seen in Figure 5.6, slight deviations are still observed though the distribution of data-points appears to follow a more linear trend after log-transformation.

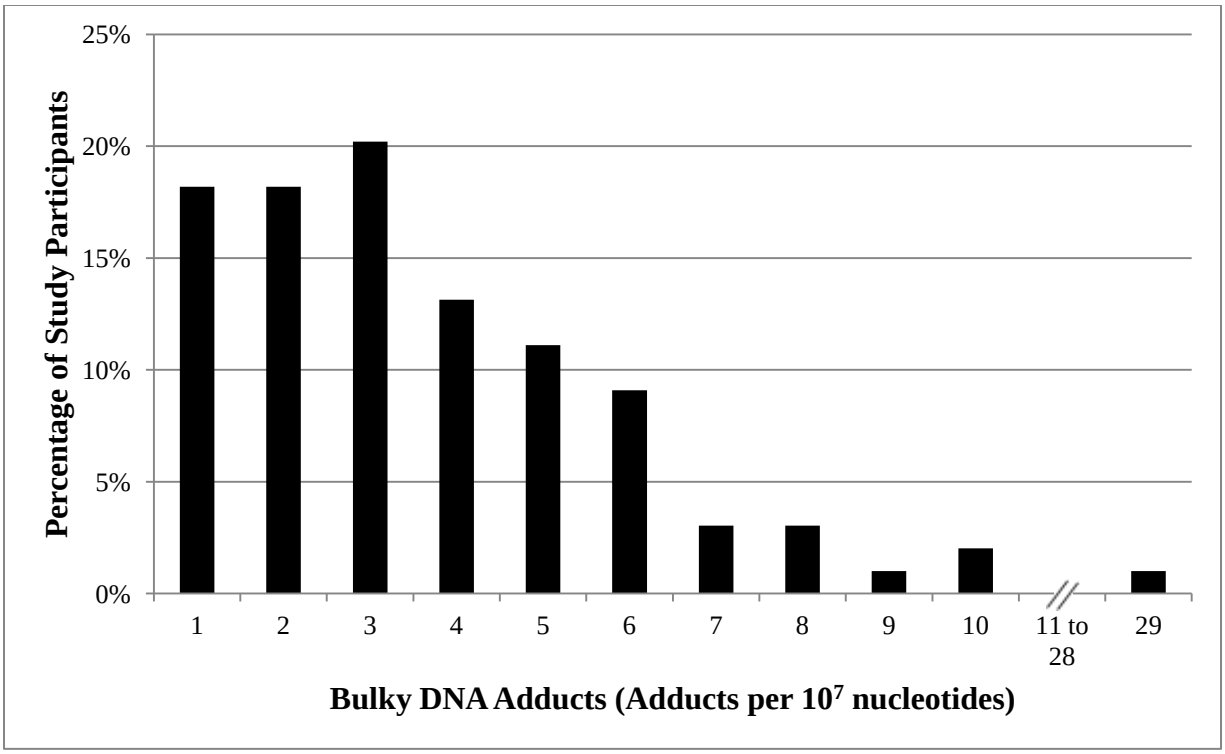


Figure 5.4: Distribution of bulky DNA adducts in blood leukocytes in Phase 1 (N=99). Midpoint of each interval is indicated.

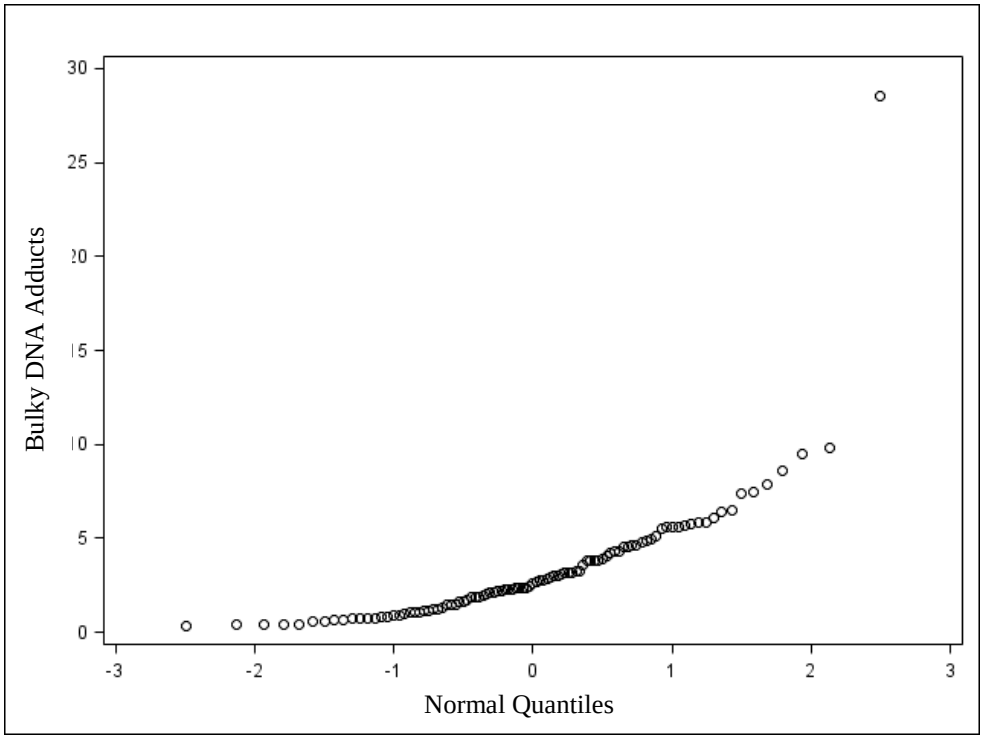


Figure 5.5: Q-Q plot of bulky DNA adducts in blood leukocytes in Phase 1 (N=99)

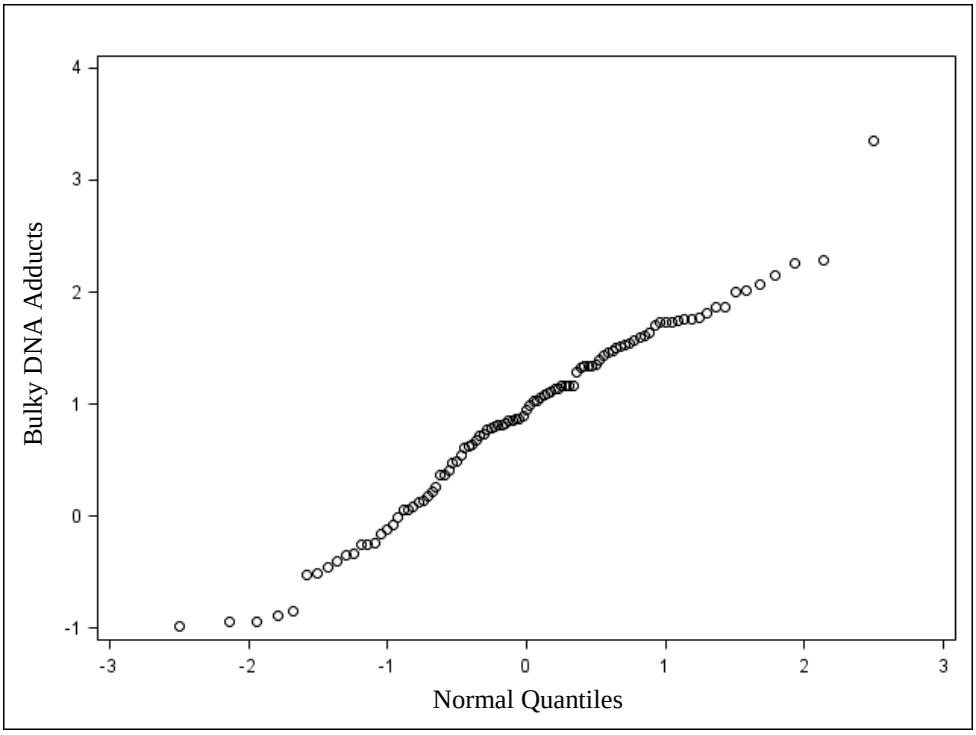


Figure 5.6: Q-Q plot of log-tranformed bulky DNA adducts in blood leukocytes in Phase 1 (N=99)

5.2.3 Covariates

The frequency distribution and percentages for covariates are presented in Table 5.2 for categorical representations and Table 5.3 presents the distribution for continuous representations. The total sample size of Phase 1 included 99 participants with a greater proportion of females (52%) than males (49%). Age was divided into approximately 10-year intervals with the highest number of participants among those between the age of 25 to 34 years (38%) followed by those between 45 and 54 (27%), 35 and 44 (24%) and those greater than 55 (10%). Overall, the study population was predominately White (75%), light drinkers (61%), never smokers (81%), never exposed to environmental tobacco smoke (90%) and with a body mass index (BMI) in the under/normal weight category (53%).

Table 5.2. Descriptive statistics for categorical covariates (N=99)

Covariate	Categories	N	Percent
Sex	Female	51	51.5
	Male	48	48.5
Age (years)	25 to 34	38	38.4
	35 to 44	24	24.2
	45 to 54	27	27.3
	≥55	10	10.1
Ethnicity	White	74	74.7
	Other	25	25.3
Alcohol Consumption[1]	Abstainers	15	15.2
	Light	60	60.6
	Moderate	24	24.2
	Heavy	0	0.0
Smoking Status	Never	80	80.8
	Past	14	14.1
	Current	5	5.1
Environmental Tobacco Smoke	Unexposed	89	89.9
	Exposed	10	10.1
BMI[2]	Underweight/Normal weight	52	52.5
	Overweight	31	31.3
	Obese	16	16.2
Fruit and Vegetable Intake (servings per day)	<3.0	19	19.2
	3.0 to <4.2	29	29.3
	4.2 to <5.9	28	29.3
	≥5.9	23	23.2

[1]Alcohol consumption categories included "Abstainers", Light (0 to ≤13.6 g/day), Moderate (13.7 to ≤54.4 g/day) and Heavy (>54.4 g/day)
[2]BMI categories included Underweight (<18.50 kg/m^2), Normal (18.50 to <25.00 kg/m^2), Overweight (25.00 to <30.00 kg/m^2) and Obese (≥30.00 kg/m^2)

Table 5.3. Descriptive statistics for continuous covariates

Covariate	Mean	SD	25th Percentile	Median	75th Percentile
Age (years)	39.4	10.3	30.0	39.0	47.0
Alcohol Consumption (g/day)	7.5	7.3	1.9	7.8	10.6
Fruit and Vegetable Intake (servings/day)	4.7	2.3	3.1	4.3	5.7

5.2.4 Covariate Representation

For continuous covariates, variable representation was selected based on balancing three priorities: 1) parsimonious number of parameters; 2) appropriate representation and; 3) interpretable effects. To determine the appropriate representations, the presence of non-linear associations was determined by fitting quadratic terms in the bivariate regression models predicting bulky DNA adduct levels. Table 5.4 presents the p-value of the partial F-statistic obtained from polynomial regression; specifically, the partial F-Statistic was used to assess whether the addition of the quadratic term significantly contributed to the prediction of bulky DNA adduct levels. No evidence of a non-linear association was observed for age and thus continuous representations were utilized. Alcohol consumption, and fruit and vegetable intake had evidence of a non-linear trend ($p<0.10$ for the partial F-statistic of the quadratic terms) and thus, categorical representations were utilized in subsequent analyses.

Table 5.4. Assessment of linear trend for continuous covariates

Covariate	Model	p[1]
Age	Covariate + Covariate^2	0.66
Alcohol	Covariate + Covariate^2	0.05
Fruit and Vegetable Intake	Covariate + Covariate^2	0.08

[1]p-value obtained from a partial F-statistic testing the addition of the quadratic term to the model

5.2.5 Genetic Factors

The frequency distribution and percentages for all genetic factors are presented in Table 5.5. The genotype distributions were all consistent with the published literature among

Caucasians (2-14). All polymorphisms were in accordance with the expected Hardy-Weinberg Equilibrium distributions (p>0.05).

Table 5.5. Frequency of genetics factors of interest and Chi-Square test of Hardy-Weinberg Equilibrium distributions

Gene	rs	Genotype Category	N	Percentage	p[1]
CYP1A1*2A	4646903	TT	71	71.7	0.66
		CT	25	25.3	
		CC	3	3.0	
CYP1A2*1F	762551	AA	46	46.5	0.76
		AC	42	42.4	
		CC	11	11.1	
CYP1B1*2	10012	CC	44	44.4	0.21
		CG	48	48.5	
		GG	7	7.1	
CYP1B1*2	1056827	GG	44	44.4	0.21
		GT	48	48.5	
		TT	7	7.1	
NAT2	1041983	CC	44	44.5	0.77
		CT	43	43.4	
		TT	12	12.1	
NAT2	1801280	TT	38	38.4	0.72
		CT	48	48.5	
		CC	13	13.1	
XPA	1800975	CC	46	46.5	0.97
		CT	40	40.4	
		TT	13	13.1	
XPD	13181	TT	49	49.5	0.56
		GT	43	43.4	
		GG	7	7.1	
XPD	1799793	CC	51	51.5	0.70
		CT	39	39.4	
		TT	9	9.1	
XRCC1	25487	CC	39	39.4	0.84
		CT	47	47.5	
		TT	13	13.1	

[1]p-value obtained from a χ^2 test

5.3 Covariate Associations with Bulky DNA Adduct Levels

Each covariate was regressed individually on log-transformed bulky DNA adduct levels (expressed as adducts per 10^7 nucleotides). For smoking status, analysis considered both Current and Past Smokers as separate categories and then grouped as one category due to small cell size. Table 5.6 present the geometric means and corresponding 95% confidence intervals (95% CI) for each category of covariates; the p-value obtained from the regression models of the log-transformed outcome was used to determine the statistical significance of the relationship. A difference in geometric mean levels of DNA adducts was observed for ethnicity where participants who self-reported as "Other" had higher mean adduct levels than those who self-identified as being "White" (p=0.04). Table 5.7 presents the continuous representation of age individually regressed on bulky DNA adduct levels; no association was observed.

Table 5.6. Demographic, anthropometric and lifestyle predictors of bulky DNA adducts in blood leukocytes

Covariate	Categories	N	Geometric Mean Adduct Levels in adducts per 10^7 nucleotides (95% CI)	p[1]
Sex	Female	51	2.46 (1.93, 3.13)	0.68
	Male	48	2.29 (1.79, 2.92)	
Ethnicity	White	74	2.14 (1.77, 2.61)	0.04
	Other	25	3.19 (2.29, 4.44)	
Alcohol Consumption[2]	Abstainers	15	2.56 (1.80, 3.63)	0.69
	Light	60	2.25 (1.80, 2.80)	
	Moderate	24	2.66 (1.72, 4.14)	
Smoking Status	Never	80	2.44 (2.01, 2.94)	0.66
	Past	14	1.95 (1.25, 3.10)	
	Current	5	2.59 (1.21, 5.53)	
Smoking Status	Never	80	2.44 (2.01, 2.94)	0.50
	Past/Current	19	2.12 (1.43, 3.10)	
Environmental Tobacco Smoke	Unexposed	89	2.36 (1.79, 2.83)	0.74
	Exposed	10	2.59 (1.51, 4.44)	
BMI[3]	Under/Normal weight	52	2.83 (2.25, 3.56)	0.09
	Overweight	31	1.88 (1.39, 2.53)	
	Obese	16	2.12 (1.40, 3.22)	
Fruit and Vegetable Intake (servings per day)	<3.0	19	3.16 (2.14, 4.62)	0.22
	3.0 to <4.2	28	2.56 (1.86, 3.53)	
	4.2 to <5.9	29	1.90 (1.38, 2.59)	
	≥5.9	23	2.29 (1.62, 3.25)	

[1]p-value obtained from a t-test
[2]Alcohol consumption categories included "Abstainers", Light (0 to ≤13.6 g/day) and Moderate (13.7 to ≤54.4 g/day)
[3]BMI categories included Underweight (<18.50 kg/m^2), Normal (18.50 to <25.00 kg/m^2), Overweight (25.00 to <30.00 kg/m^2) and Obese (≥30.00 kg/m^2)

Table 5.7. Continuous representation of age on bulky DNA adducts in blood leukocytes

Exposure	Parameter Estimate	Percent Change in Bulky DNA Adduct Levels per unit Increase in Exposure	p[1]
Age (per 10 years)	-0.12	-11.34%	0.15

[1]p-value obtained from a t-test

5.4 Covariate Associations with Dietary Heterocyclic Aromatic Amine Intake

The relationships between dietary HAAs and categorical covariates were examined to provide an understanding of their relationships and inform on the potential for confounding. Bivariate associations were tested by the Chi-Square test and Fisher's exact test to determine whether the distribution of exposures of interest differed according to covariates (Table 5.8-5.10). For ease of presentation, dichotomous representations of dietary HAAs based on combining equal-distant Categories 1 and 2, and Categories 3 and 4 were utilized. A statistically significant relationship defined at a 0.05 level of significance was observed between dietary DiMeIQx and environmental tobacco smoke. Table 5.11 presents the results for age and their associations with dietary HAAs evaluated using t-tests, no significant associations were observed. Table 5.12 presents the Spearman correlation coefficients for dietary HAA intake; PhIP, MeIQx and DiMeIQx were found to be highly correlated with each other.

Table 5.8. Frequency of categorical covariates among quartiles of dietary PhIP

Covariate	Categories	Categories 1 and 2 PhIP≤165.68 ng/day		Categories 3 and 4 PhIP>165.68 ng/day		p[1]
		N	%	N	%	
Sex	Male	26	46.4	22	51.2	0.64
	Female	30	53.6	21	48.8	
Ethnicity	White	40	71.4	34	79.1	0.39
	Other	16	28.6	9	20.9	
Alcohol Consumption[2]	Abstainers	8	14.3	7	16.3	0.68
	Light	36	64.3	24	55.8	
	Moderate	12	21.4	12	27.9	
Smoking Status	Never	46	83.6	33	76.7	0.64
	Past	7	12.7	7	16.3	
	Current	2	3.6	3	7.0	
Environmental Tobacco Smoke	Unexposed	51	91.1	38	88.4	0.66
	Exposed	5	8.9	5	11.6	
BMI[3]	Under/Normal weight	34	60.7	18	41.9	0.17
	Overweight	14	25.0	17	39.5	
	Obese	8	14.3	8	18.6	
Fruit and Vegetable Consumption (servings per day)	<3.0	12	21.4	7	16.3	0.83
	3.0 to <4.2	14	25.0	14	32.6	
	4.2 to <5.9	17	30.4	12	27.9	
	≥5.9	13	23.2	10	23.3	

[1]p-value obtained from a χ^2 test
[2]Alcohol consumption categories included "Abstainers", Light (0 to ≤13.6 g/day), Moderate (13.7 to ≤54.4 g/day) and Heavy (>54.4 g/day)
[3]BMI categories included Underweight (<18.50 kg/m^2), Normal (18.50 to <25.00 kg/m^2), Overweight (25.00 to <30.00 kg/m^2) and Obese (≥30.00 kg/m^2)

Table 5.9. Frequency of categorical covariates among quartiles of dietary MeIQx

Covariate	Categories	Categories 1 and 2 MeIQx≤31.09 ng/day		Categories 3 and 4 MeIQx>31.09 ng/day		p[1]
		N	%	N	%	
Sex	Male	22	41.5	26	56.5	0.14
	Female	31	58.5	20	43.5	
Ethnicity	White	37	69.8	37	80.4	0.23
	Other	16	30.2	9	19.6	
Alcohol Consumption[2]	Abstainers	9	17.0	6	13.0	0.40
	Light	34	64.1	26	56.5	
	Moderate	10	18.9	14	30.4	
Smoking Status	Never	45	84.9	34	75.5	0.50
	Past	6	11.3	8	17.8	
	Current	2	3.8	3	6.7	
Environmental Tobacco Smoke	Unexposed	50	94.3	39	84.8	0.12
	Exposed	3	5.7	7	15.2	
BMI[3]	Under/Normal weight	31	58.5	21	45.6	0.42
	Overweight	15	28.3	16	34.8	
	Obese	7	13.2	9	19.6	
Fruit and Vegetable Consumption (servings per day)	<3.0	10	18.9	9	19.6	0.99
	3.0 to <4.2	15	28.3	13	28.3	
	4.2 to <5.9	16	30.2	13	28.2	
	≥5.9	12	22.6	11	23.9	

[1]p-value obtained from a χ^2 test
[2]Alcohol consumption categories included "Abstainers", Light (0 to ≤13.6 g/day), Moderate (13.7 to ≤54.4 g/day) and Heavy (>54.4 g/day)
[3]BMI categories included Underweight (<18.50 kg/m^2), Normal (18.50 to <25.00 kg/m^2), Overweight (25.00 to <30.00 kg/m^2) and Obese (≥30.00 kg/m^2)

Table 5.10. Frequency of categorical covariates among quartiles of dietary DiMeIQx

Covariate	Categories	Categories 1 and 2 DiMeIQx≤6.48 ng/day		Categories 3 and 4 DiMeIQx>6.48 ng/day		p[1]
		N	%	N	%	
Sex	Male	23	45.1	25	52.1	0.49
	Female	28	54.9	23	47.9	
Ethnicity	White	36	70.6	38	79.2	0.33
	Other	15	29.4	10	20.8	
Alcohol Consumption[2]	Abstainers	8	15.7	7	14.6	0.93
	Light	30	58.8	30	62.5	
	Moderate	13	25.5	11	22.9	
Smoking Status	Never	43	84.3	36	76.6	0.32
	Past	7	13.7	7	14.9	
	Current	1	2.0	4	8.5	
Environmental Tobacco Smoke	Unexposed	49	96.1	40	83.3	0.04
	Exposed	2	3.9	8	16.7	
BMI[3]	Under/Normal weight	31	60.8	21	43.8	0.13
	Overweight	15	29.4	16	33.3	
	Obese	5	9.8	11	22.9	
Fruit and Vegetable Consumption (servings per day)	<3.0	10	19.6	9	18.8	0.09
	3.0 to <4.2	10	19.6	18	37.5	
	4.2 to <5.9	20	39.2	9	18.7	
	≥5.9	11	21.6	12	25.0	

[1]p-value obtained from a χ^2 test
[2]Alcohol consumption categories included "Abstainers", Light (0 to ≤13.6 g/day), Moderate (13.7 to ≤54.4 g/day) and Heavy (>54.4 g/day)
[3]BMI categories included Underweight (<18.50 kg/m^2), Normal (18.50 to <25.00 kg/m^2), Overweight (25.00 to <30.00 kg/m^2) and Obese (≥30.00 kg/m^2)

Table 5.11. Mean and standard deviation of age among categories of PhIP, MeIQx and DiMeIQx

Covariate		Categories 1 and 2 μ (95% CI)	Categories 3 and 4 μ (95% CI)	p[1]
PhIP				
	Age (years)	38.2 (35.5, 40.9)	41.1 (38.0, 44.1)	0.17
MeIQx				
	Age (years)	38.2 (35.4, 40.9)	40.9 (37.9, 43.9)	0.19
DiMeIQx				
	Age (years)	38.5 (35.6, 41.4)	40.4 (37.5, 43.4)	0.35

[1]p-value obtained from a t-test

Table 5.12. Spearman correlation between dietary HAAs

$r_{Spearman}$ (p-value)[1]	PhIP	MeIQx	DiMeIQx
PhIP	1	0.67 (<0.01)	0.66 (<0.01)
MeIQx		1	0.80 (<0.01)
DiMeIQx			1

[1]p-value obtained from Spearman rank correlation

5.5 Main Analysis

The relationships between dietary exposure to HAAs (PhIP, MeIQx and DiMeIQx), and bulky DNA adduct levels were examined using least squares regression. Exposure conceptualization included continuous and categorical representations via equal-distant categories and dichotomization based on the 75th percentile. The bivariate associations between dietary PhIP, MeIQx, and DiMeIQx, represented continuously, and quantities of bulky DNA adducts are presented in Table 5.13. For continuous representations, no significant associations were observed between exposure to PhIP, MeIQx and DiMeIQx, on bulky DNA adduct levels. The main effects of PhIP, MeIQx and DiMeIQx represented as equal-distant categories are presented in Table 5.14. None of the specific HAA intakes were significantly associated with bulky DNA adduct levels and evidence of non-linear associations were observed. In the main effects analysis using dichotomous representations of dietary HAA intakes based on the 75th

percentile, no statistically significant associations were observed (Table 5.15). However, for PhIP, higher mean bulky DNA adduct levels were observed among those with higher versus lower intakes of dietary PhIP (p=0.16).

Table 5.13. Analysis of standardized dietary HAA intake and log-transformed levels of bulky DNA adducts in blood leukocytes.

HAA	Parameter Estimate	Percent Change in Bulky DNA adduct Level per SD Increase in HAA Intake	p[1]
PhIP (per SD)	0.006	0.61%	0.94
MeIQx (per SD)	-0.02	-2.21%	0.80
DiMeIQx (per SD)	-0.07	-6.95%	0.41

[1]p-value obtained from a t-test

Table 5.14. Analysis of dietary HAA intake (represented as equal-distant categories) and levels of bulky DNA adducts in blood leukocytes.

HAA	Categories (ng/day)	N	Geometric Mean Adduct Levels in adducts per 10^7nucleotides (95% CI)	p[1]
PhIP	Category 1: ≤82.84	26	2.36 (1.70, 3.29)	0.21
	Category 2: 82.85 to 165.68	30	2.48 (1.82, 3.39)	
	Category 3: 165.69 to 248.51	18	1.68 (1.13, 2.48)	
	Category 4: >248.51	25	2.92 (2.10, 4.10)	
MeIQx	Category 1: ≤15.55	26	2.20 (1.58, 3.10)	0.79
	Category 2: 15.56 to 31.09	27	2.72 (1.95, 3.78)	
	Category 3: 31.10 to 46.64	21	2.18 (1.49, 3.16)	
	Category 4: >46.64	25	2.39 (1.70, 3.35)	
DiMeIQx	Category 1: ≤3.24	23	2.89 (2.03, 4.10)	0.31
	Category 2: 3.25 to 6.48	28	1.88 (1.36, 2.59)	
	Category 3: 6.49 to 9.72	24	2.41 (1.70, 3.39)	
	Category 4: >9.72	24	2.56 (1.82, 3.60)	

[1]p-value obtained from a t-test

Table 5.15. Analysis of dietary HAA intake (represented as dichotomized based on the 75th percentile) and levels of bulky DNA adducts in blood leukocytes.

HAA	Categories (ng/day)	N	Geometric Mean Adduct Levels in adducts per 10^7 nucleotides (95% CI)	p[1]
PhIP	Category 1, 2 and 3: ≤248.51	74	2.23 (1.82, 2.69)	0.16
	Category 4: >248.51	25	2.92 (2.08, 4.10)	
MeIQx	Category 1, 2, and 3: ≤46.64	74	2.36 (1.93, 2.89)	0.97
	Category 4: >46.64	25	2.39 (1.70, 3.35)	
DiMeIQx	Category 1, 2 and 3: ≤9.72	75	2.32 (1.90, 2.83)	0.62
	Category 4: >9.72	24	2.56 (1.80, 3.63)	

[1]p-value obtained from a t-test

5.6 Multivariable Analysis

Stepwise selection was used to create a parsimonious model of covariates that were predictive of bulky DNA adducts using a liberal p-value of 0.20. Due to observations of non-linear associations between dietary HAA intakes and bulky DNA adduct levels, continuous representation of dietary HAAs was not further assessed. The covariates considered included sex, age, ethnicity, alcohol consumption, smoking status, exposure to environmental tobacco smoke, BMI, and fruit and vegetable consumption. Ethnicity, BMI and fruit and vegetable consumption were predictors of bulky DNA adduct levels at a 0.20 level of significance. The geometric mean adduct levels for identified predictors of bulky DNA adducts are presented in Table 5.16.

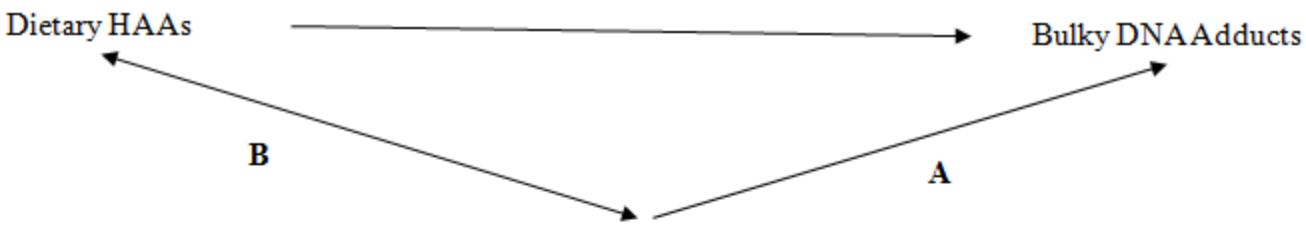
Participants who self-reported their ethnicity as “Other” had a non-significantly higher mean adduct level than those who self-identified as being “White.” The under/normal weight BMI category was associated with the highest mean DNA adduct level (p=0.05) and a general non-significant decrease in DNA adduct level was observed with increasing fruit and vegetable intake (p=0.09).

Table 5.16. Multivariable analysis of predictors of bulky DNA adduct levels in blood leukocytes

Predictor	**Categories**	**N**	**Geometric Mean Adduct Levels in adducts per 10^7 nucleotides (95% CI)**	**p[1]**
Ethnicity	White	74	2.14 (1.72, 2.64)	0.18
	Other	25	2.77 (1.97, 3.94)	
BMI[2]	Under/Normal weight	52	3.19 (2.51, 4.06)	0.05
	Overweight	31	1.99 (1.43, 2.75)	
	Obese	16	2.29 (1.51, 3.49)	
Fruit and Vegetable Intake (servings per day)	<3.0	19	3.46 (2.34, 5.05)	0.09
	3.0 to <4.2	28	2.59 (1.86, 3.60)	
	4.2 to <5.9	29	1.84 (1.30, 2.61)	
	≥5.9	23	2.18 (1.51, 3.13)	

[1]p-value obtained from a t-test
[2]BMI categories included Underweight (<18.50 kg/m^2), Normal (18.50 to <25.00 kg/m^2), Overweight (25.00 to <30.00 kg/m^2) and Obese (≥30.00 kg/m^2)

To assess the extent to which identified predictors of bulky DNA adducts may confound the relationship between dietary HAA exposures and bulky DNA adduct levels, Figure 5.7 summarizes the following: 1) directionality of the relationship between each predictor and bulky DNA adducts and; 2) the bivariate association between each predictor and dietary HAAs. Specifically, bivariate associations between predictors of bulky DNA adducts and dietary HAAs were determined based on the 0.20 level of significance.



Predictors of Bulky DNA Adducts	A: Higher Levels of Bulky DNA Adducts[1]	B: Higher Intake of Dietary PhIP[2]	B: Higher Intake of Dietary MeIQx[2]	B: Higher Intake of Dietary DiMeIQx[2]
Ethnicity	Non-White ethnic group	No Association	No Association	No Association
BMI	Lower BMI	Higher BMI	No Association	Higher BMI
Fruit and Vegetable Intake	Lower Intake	No Association	No Association	Non-linear Trend

[1]Directionality of relationship between predictors and bulky DNA adducts is based on a multivariable least squares regression analysis
[2]Directionality of relationship between predictors and dietary HAAs is based on bivariate associations tested using the χ^2 test; an association is defined by $p<0.20$ level of significance

Figure 5.7: Evaluation of the extent of to which predictors of bulky DNA adducts in blood leukocytes may confound the relationships between dietary HAAs and bulky DNA adduct levels.

Multivariable analysis of dietary PhIP, MeIQx and DiMeIQx while controlling for the identified predictors of bulky DNA adducts in each model is presented in Table 5.17. No statistically significant associations were observed between dietary HAA intake and bulky DNA adduct levels in both equal-distant and dichotomous representations. However, a general increase in bulky DNA adduct levels was observed with higher intakes of dietary PhIP; those with greater than 248.51 ng/day dietary PhIP intake (Category 4) had the highest mean adduct levels.

Table 5.17. Multivariable analysis of specific dietary HAAs and bulky DNA adducts levels in blood leukocytes.

Predictor	**Categories (ng/day)**	**N**	**Geometric Mean Adduct Levels in adducts per 10^7 nucleotides[1] (95% CI)**	**p[2]**
PhIP	Category 1: ≤82.84	26	2.25 (1.60, 3.16)	0.30
	Category 2: 82.85 to 165.68	30	2.46 (1.77, 3.46)	
	Category 3: 165.69 to 248.51	18	1.90 (1.25, 2.89)	
	Category 4: >248.51	25	3.06 (2.16, 4.35)	
	Category 1, 2 and 3: ≤248.51	74	2.25 (1.77, 2.83)	0.10
	Category 4: >248.51	25	3.06 (2.18, 4.31)	
MeIQx	Category 1: ≤15.55	26	2.12 (1.52, 2.97)	0.61
	Category 2: 15.56 to 31.09	27	2.86 (1.97, 4.14)	
	Category 3: 31.10 to 46.64	21	2.34 (1.55, 3.49)	
	Category 4: >46.64	25	2.61 (1.86, 3.71)	
	Category 1, 2, and 3: ≤46.64	74	2.39 (1.88, 3.03)	0.67
	Category 4: >46.64	25	2.59 (1.84, 3.63)	
DiMeIQx	Category 1: ≤3.24	23	2.75 (1.93, 3.94)	0.33
	Category 2: 3.25 to 6.48	28	1.90 (1.32, 2.72)	
	Category 3: 6.49 to 9.72	24	2.36 (1.62, 3.46)	
	Category 4: >9.72	24	2.75 (1.93, 3.90)	
	Category 1, 2 and 3: ≤9.72	75	2.32 (1.84, 2.94)	0.36
	Category 4: >9.72	24	2.77 (1.95, 3.94)	

[1]Adjusted for ethnicity, BMI, and fruit and vegetable consumption
[2]p-value obtained from a t-test

5.7 Genetic Analysis

5.7.1 Genetic Determinants of Bulky DNA Adduct Levels in Blood Leukocytes

For genetic factors, Table 5.18 presents the geometric mean bulky DNA adduct level and corresponding 95% CI for each genetic factor while adjusting for predictors of bulky DNA adducts. Genetic categorization was described in detail in Chapter 4.1.7. A statistically significant association was observed for NAT1; specifically, those with one or more alleles conferring the putative fast acetylator phenotype had a lower DNA adduct level than those with a slow acetylator phenotype (p=0.02).

Table 5.18. Multivariable analysis of genetic predictors of bulky DNA adduct levels in blood leukocytes

Gene	rs	Genotype Category	N	Geometric Mean Adduct Levels in adducts per 10^7 nucleotides[1] (95% CI)	p[2]
CYP1A1*2A	4646903	CC/CT	28	2.69 (1.93, 3.74)	0.46
		TT	71	2.32 (1.80, 2.97)	
CYP1A2*1F	762551	AA	53	2.46 (1.86, 3.22)	0.97
		AC/CC	46	2.44 (1.86, 3.19)	
CYP1B1*2	10012; 1056827	CG/GG+GT/TT	55	2.20 (1.72, 2.83)	0.16
		CC+GG	44	2.80 (2.12, 3.71)	
NAT1	1057126; 15561	1+	51	2.10 (1.63, 2.66)	0.02
		0	47	3.13 (2.34, 4.14)	
NAT2	1041983; 1801280	0, 1	48	2.29 (1.75, 2.97)	0.40
		2+	51	2.61 (1.99, 3.53)	
XPA	1800975	CC	53	2.86 (2.16, 3.74)	0.10
		CT/TT	46	2.16 (1.67, 2.77)	
XPD	13181	CC/CA	50	2.51 (1.93, 3.29)	0.73
		AA	49	2.36 (1.80, 3.10)	
XPD	1799793	AA/GA	48	2.51 (1.92, 3.39)	0.84
		GG	51	2.36 (1.80, 3.10)	
XRCC1	25487	AA/GA	60	2.66 (2.10, 3.42)	0.17
		GG	39	2.10 (1.54, 2.83)	

[1]Adjusted for ethnicity, BMI, and fruit and vegetable consumption
[2]p-value obtained from a t-test

5.7.2 Gene-Diet Interaction Analysis

For gene-diet interactions, all HAAs were dichotomized by combining categories 3 and 4 and using categories 1 and 2 as the referent category. The effects of HAAs on bulky DNA adduct levels within strata of genetic factors were assessed while controlling for predictors identified in multivariable analyses (Section 5.6). Tables 5.19 to 5.21 present the gene-diet interaction results assessed for PhIP, MeIQx and DiMeIQx on bulky DNA adduct levels.

NAT1 polymorphisms were found to modify the relationships between dietary PhIP, MeIQx and DiMeIx and bulky DNA adduct levels (PhIP: p=0.01; MeIQx: p=0.03; DiMeIQx: p=0.03). Specifically, among those with one or more alleles of the putative fast acetylator phenotype, higher intakes of dietary HAAs did not have an appreciable change in mean DNA adduct levels. However, among slow acetylators (those without any rapid acetylator allele) higher intakes of dietary HAAs were associated with higher DNA adduct levels compared to those with lower intakes.

Table 5.19. Gene-PhIP interactions on log-transformed bulky DNA adduct levels in blood leukocytes

Gene	rs	Genotype Category	Categories 1 and 2 PhIP≤165.68 ng/day Geometric Mean Adduct Levels (95% CI)[1]	Categories 3 and 4 PhIP>165.68 ng/day Geometric Mean Adduct Levels (95% CI)[1]	p[2]
CYP1A1*2A	4646903	CC/CT	3.10 (2.08, 4.62)	2.03 (1.17, 3.49)	0.08
		TT	2.01 (1.46, 2.77)	2.64 (1.93, 3.63)	
CYP1A2*1F	762551	AA	2.27 (1.63, 3.16)	2.86 (1.84, 4.44)	0.43
		AC/CC	2.51 (1.75, 3.63)	2.39 (1.67, 3.39)	
CYP1B1*2	10012;	CG/GG+GT/TT	2.05 (1.52, 2.77)	2.48 (1.68, 3.71)	0.27
	1056827	CC+GG	3.10 (2.08, 4.57)	2.56 (1.79, 3.67)	
NAT1	1057126;	1+	2.36 (1.75, 3.19)	1.68 (1.17, 2.41)	0.01
	15561	0	2.41 (1.67, 3.46)	4.14 (2.83, 6.05)	
NAT2	1041983;	0, 1	2.32 (1.67, 3.25)	2.18 (1.48, 3.22)	0.44
	1801280	2+	2.41 (1.68, 3.46)	2.94 (2.01, 4.31)	
XPA	1800975	CC	2.41 (1.67, 3.49)	3.32 (2.32, 4.81)	0.12
		CT/TT	2.32 (1.70, 3.16)	1.88 (1.30, 2.77)	
XPD	13181	CC/CA	2.56 (1.82, 3.56)	2.44 (1.65, 3.60)	0.50
		AA	2.16 (1.52, 3.10)	2.61 (1.79, 3.82)	
XPD	1799793	AA/GA	2.36 (1.67, 3.35)	2.69 (1.79, 4.06)	0.77
		GG	2.36 (1.68, 3.32)	2.44 (1.70, 3.49)	
XRCC1	25487	AA/GA	2.41 (1.77, 3.32)	3.00 (2.14, 4.18)	0.23
		GG	2.27 (1.55, 3.35)	1.86 (1.20, 2.89)	

[1]Geometric mean adduct levels in adducts per 10^7 nucleotides adjusted for ethnicity, BMI, and fruit and vegetable consumption
[2]p-value for interaction term

Table 5.20. Gene-MeIQx interactions on log-transformed bulky DNA adduct levels in blood leukocytes

Gene	rs	Genotype Category	Categories 1 and 2 MeIQx≤31.09 ng/day Geometric Mean Adduct Levels (95% CI)[1]	Categories 3 and 4 MeIQx>31.09 ng/day Geometric Mean Adduct Levels (95% CI)[1]	p[2]
CYP1A1*2A	4646903	CC/CT	2.77 (1.86, 4.10)	2.51 (1.42, 4.44)	0.68
		TT	2.25 (1.62, 3.10)	2.39 (1.75, 3.29)	
CYP1A2*1F	762551	AA	2.29 (1.60, 3.32)	2.61 (1.79, 3.78)	0.50
		AC/CC	2.53 (1.80, 3.56)	2.29 (1.57, 3.32)	
CYP1B1*2	10012;	CG/GG+GT/TT	2.16 (1.58, 2.97)	2.27 (1.57, 3.25)	0.67
	1056827	CC+GG	2.94 (2.01, 4.35)	2.66 (1.84, 4.86)	
NAT1	1057126;	1+	2.41 (1.79, 3.29)	1.63 (1.13, 2.36)	0.03
	15561	0	2.59 (1.77, 3.74)	3.63 (2.56, 5.21)	
NAT2	1041983;	0, 1	2.39 (1.72, 3.35)	2.12 (1.43, 3.13)	0.53
	1801280	2+	2.51 (1.72, 3.67)	2.77 (1.92, 3.97)	
XPA	1800975	CC	2.69 (1.86, 3.90)	3.00 (2.05, 4.35)	0.56
		CT/TT	2.25 (1.62, 3.10)	2.03 (1.42, 2.94)	
XPD	13181	CC/CA	2.69 (1.92, 3.82)	2.27 (1.54, 3.32)	0.29
		AA	2.16 (1.51, 3.10)	2.61 (1.80, 3.78)	
XPD	1799793	AA/GA	2.59 (1.80, 3.74)	2.39 (1.62, 3.49)	0.62
		GG	2.32 (1.63, 3.25)	2.51 (1.75, 3.63)	
XRCC1	25487	AA/GA	2.53 (1.86, 3.49)	2.83 (2.01, 3.97)	0.46
		GG	2.25 (1.51, 3.35)	1.93 (1.28, 2.94)	

[1]Geometric mean adduct levels in adducts per 10^7 nucleotides adjusted for ethnicity, BMI, and fruit and vegetable consumption
[2]p-value for interaction term

Table 5.21. Gene-DiMeIQx interactions on log-transformed bulky DNA adduct levels in blood leukocytes

Gene	rs	Genotype Category	Categories 1 and 2 DiMeIQx≤6.48 ng/day Geometric Mean Adduct Levels (95% CI)[1]	Categories 3 and 4 DiMeIQx>6.48 ng/day Geometric Mean Adduct Levels (95% CI)[1]	p[2]
CYP1A1*2A	4646903	CC/CT	2.89 (1.88, 4.44)	2.41 (1.49, 3.94)	0.25
		TT	2.05 (1.48, 2.83)	2.64 (1.92, 3.63)	
CYP1A2*1F	762551	AA	2.16 (1.51, 3.10)	2.83 (1.92, 4.14)	0.43
		AC/CC	2.41 (1.68, 3.49)	2.41 (1.70, 3.46)	
CYP1B1*2	10012;	CG/GG+GT/TT	2.05 (1.51, 2.83)	2.44 (1.67, 3.53)	0.62
	1056827	CC+GG	2.83 (1.84, 4.31)	2.77 (1.93, 3.97)	
NAT1	1057126;	1+	2.25 (1.65, 3.06)	1.75 (1.21, 2.53)	0.03
	15561	0	2.25 (1.49, 3.39)	3.82 (2.69, 5.42)	
NAT2	1041983;	0, 1	2.41 (1.72, 3.42)	2.10 (1.45, 3.03)	0.13
	1801280	2+	2.16 (1.48, 3.19)	2.13 (2.18, 4.48)	
XPA	1800975	CC	2.29 (1.52, 3.42)	3.32 (2.36, 4.71)	0.12
		CT/TT	2.29 (1.67, 3.16)	1.95 (1.35, 2.83)	
XPD	13181	CC/CA	2.39 (1.67, 3.46)	2.61 (1.80, 3.78)	0.85
		AA	2.20 (1.54, 3.16)	2.56 (1.75, 3.74)	
XPD	1799793	AA/GA	2.23 (1.54, 3.25)	2.77 (1.90, 4.06)	0.62
		GG	2.34 (1.63, 3.35)	2.46 (1.72, 3.49)	
XRCC1	25487	AA/GA	2.41 (1.32, 3.03)	3.03 (2.14, 4.26)	0.65
		GG	1.99 (1.28, 3.10)	2.14 (1.45, 3.16)	

[1]Geometric mean adduct levels in adducts per 10^7 nucleotides adjusted for ethnicity, BMI, and fruit and vegetable consumption
[2]p-value for interaction term

5.8 Additional Analysis

5.8.1. Sex Specific Analysis

An analysis was conducted to evaluate sex-specific associations between dietary HAA intakes and bulky DNA adduct levels (Table 5.22); no differences were observed between males and females.

Table 5.22. Dietary HAA intake on log-transformed bulky DNA adduct levels in blood leukocytes by sex

Gene	Category (ng/day)	Females Geometric Mean Adduct Levels (95% CI)[1]	Males Geometric Mean Adduct Levels (95% CI)[1]	p[2]
PhIP	Categories 1 and 2 (PhIP≤165.68)	2.14 (1.49- 3.06)	2.77 (1.88-4.14)	0.25
	Categories 3 and 4 (PhIP>165.68)	2.29 (1.58-3.35)	2.64 (1.88-3.67)	
MeIQx	Categories 1 and 2 (MeIQx≤31.09)	2.29 (1.62-3.25)	2.64 (1.82-3.78)	0.52
	Categories 3 and 4 (MeIQx>31.09)	2.56 (1.68-3.86)	2.34 (1.65-3.32)	
DiMeIQx	Categories 1 and 2 (DiMeIQx≤6.48)	2.08 (1.45-2.97)	2.59 (1.80-3.71)	0.22
	Categories 3 and 4 (DiMeIQx>6.48)	2.89 (1.93-4.26)	2.36 (1.67-3.35)	

[1]Geometric mean adduct levels in adducts per 10^7 nucleotides adjusted for ethnicity, BMI, and fruit and vegetable consumption
[2]p-value obtained for the interaction term

5.9 Regression Diagnostics

Residual analysis was undertaken to assess the appropriateness of the model predicting log-transformed bulky DNA adduct levels. Observations that were identified as outliers or those with a high leverage or influence were further investigated. Specifically, each observation was reviewed for data entry errors in exposure assessment and the biologic plausibility for the range of bulky DNA adduct levels was considered. None of the identified outliers had strong leverage; thus, limiting the influence of outliers on the regression coefficient estimates. As well, the influence of these observations was further investigated by removal of each observation from the study population; no difference in study results was observed. In addition, residuals were

assessed for normality by plotting studentized residuals against predicted values and no systematic trend was observed. Overall, the evaluation of regression diagnostics provided confidence that the assumptions of least squares regression were not violated.

5.10 Summary of Phase 1 Results

Bulky DNA adduct levels in white blood cells are increasingly used in the epidemiologic literature to represent the absorbed dose of exposure to genotoxic agents such as HAAs and may be reflective of cancer risk. Phase 1 investigated the association between dietary HAAs and other individual characteristics on bulky DNA adduct levels in 99 healthy individuals. Significant differences were observed between BMI and NAT1 polymorphisms where the highest levels of DNA adducts was observed among those in the under/normal weight category of BMI and those without the NAT1 variant alleles. No main effects were found for dietary HAA exposures and bulky DNA adduct levels though there was a non-significant increase in adduct levels among those in the highest intake category of dietary PhIP. A significant interaction was observed for dietary HAAs and the NAT1 polymorphism where the highest level of bulky DNA adducts was found in the highest intake of dietary HAAs among those with the NAT1 slow acetylator phenotype.

5.11 References

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Chapter 6: Phase 2 Study Design and Methods

This doctoral dissertation investigated the relationship between heterocyclic aromatic amines (HAAs) and colorectal adenoma risk in two epidemiologic studies (Phase 1 and 2). Phase 2 examined the relationships between: a) exposure to dietary HAAs and colorectal adenoma prevalence and; b) exposure to meat mutagenicity on the risk of colorectal adenoma. Phase 2 also examined the role of polymorphisms in genes involved in the biotransformation of HAAs and DNA repair (including the repair of DNA adducts and damage induced by HAAs/DNA adducts) in modifying the HAA/meat mutagenicity-colorectal adenoma relationship. This Chapter presents an overview of the study design and methods of Phase 2.

6.1 Phase 2: Dietary HAA Intake and Risk of Colorectal Adenomas

Phase 2 of this research was funded by the Canadian Cancer Society (CCS); the goal being to contribute to a better understanding of the potential role of HAAs in colorectal carcinogenesis. Specifically, Phase 2 consisted of a cross-sectional study (n=342) nested within a larger CCS-funded clinic-based cross-sectional study investigating the prevalence of methylation markers in relation to colorectal adenomas (Principal Investigator: W. D. King). Subjects included patients undergoing a screening colonoscopy at a large endoscopy centre. Consenting patients provided a fasting blood sample and answered a questionnaire.

A dichotomous outcome was defined based on the pathology findings using standard diagnostic criteria, where participants with colorectal adenomas were assigned to the event group. Subjects with no abnormality detected at colonoscopy were assigned to the non-event group. The questionnaire for the larger study provided information on personal characteristics, and lifestyle and dietary factors. As well, biochemical factors in blood assessed in the larger study were also considered as potential confounders. Within this nested sample, the

relationships between: a) exposure to dietary HAAs and colorectal adenoma prevalence and; b) exposure to meat mutagenicity on the risk of colorectal adenoma were examined. Common polymorphisms in genes involved in the biotransformation of HAAs and DNA repair were also genotyped in DNA isolated from blood to determine effect modification by genetic susceptibility on the HAA/meat mutagenicity-colorectal adenoma relationship.

6.1.1 Study Subjects

A sample of 342 patients (N=126 diagnosed with colorectal adenoma and N=216 with no abnormality detected at colonoscopy) was selected for Phase 2 from the larger CCS-funded study, which recruited a total of 448 patients. The target population for the larger study included male and female subjects, aged 40 to 65 undergoing a screening colonoscopy at the Hotel Dieu Hospital in Kingston, Ontario. This clinic currently performs over 2000 colonoscopies per year among potentially eligible subjects. Screening colonoscopy refers to an examination of the colon in patients who currently may be at risk for developing CRC such as individuals with a positive fecal occult blood test or a positive family history of CRC. This age group was targeted as this represents a meaningful exposure time period in terms of the postulated biologic mechanisms of colorectal adenoma development.

6.1.2 Recruitment

Participants of Phase 2 were selected from consenting patients who participated in the larger CCS-funded study (Figure 6.1). Briefly, relatively healthy individuals undergoing a screening colonoscopy were being targeted for this larger study. Patients with previous inflammatory bowel disease or a diagnosis of cancer in the last 5 years (except non-melanoma skin cancer), and patients with known genetic disorders that predispose to colorectal cancer (CRC) were not eligible since it is postulated that the underlying biologic mechanisms related to

colorectal adenoma risk may be different for these patients as compared to individuals without these conditions. Similarly, subjects with any abnormality (adenoma or cancer) detected on a previous colonoscopy were excluded as the biologically relevant time period may be different for this subset of patients. As well, participants diagnosed with CRC (<5%) at the time of their colonoscopy were not included in Phase 2 due to concerns that biomarker levels of dietary covariates in these patients will not be reflective of levels prior to cancer development.

In the larger study, 668 eligible subjects were invited to participate 448 of which consented. Of those 448, 370 had a successful colonoscopy and blood draw, normal-appearing colon tissue biopsies were obtained during colonoscopy and completed questionnaires were returned. A colonoscopy was defined as unsuccessful if the examination did not reach the cecum (estimated to include about 5% of colonoscopies) or for whom the bowel was not sufficiently cleansed prior to the procedure since these situations would result in an incomplete examination of the colon and the inability to rule out the presence of an abnormality.

Phase 2 of this research included participants of the larger study who completed all data collection procedures. Twenty-eight subjects with hyperplastic polyps were not considered eligible for Phase 2 yielding a final sample size of 342 participants (Objective #1); 126 participants were newly diagnosed with a colorectal adenoma at colonoscopy and 216 participants had no abnormality detected at colonoscopy. For genetic analysis (Objective #2), 327 out of 342 subjects were eligible for genotyping based on having adequate blood DNA yields and concentrations.

Phase 2 was conceptualized and planned during the development of the larger study procedures; thus, the objectives of Phase 2 were all consistent with the informed consent given by participants of the larger study. Specifically, participants consented to having their blood

samples stored in order to examine, at a later date, other biologic and genetic markers that were also related to CRC risk (e.g. polymorphisms in genes involved in the biotransformation of HAAs and DNA repair genes).

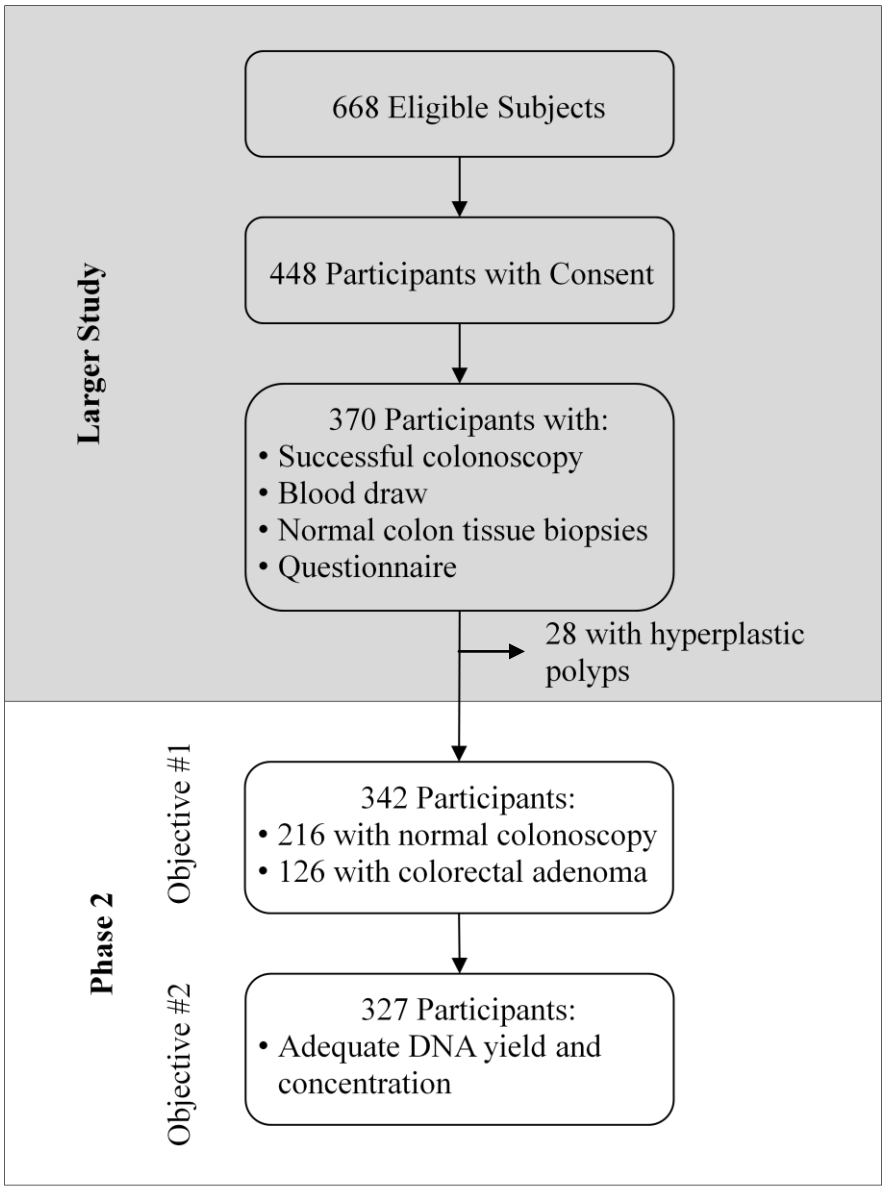

Figure 6.1: Phase 2 subject selection flowchart

6.1.3 Data Collection

Sources of information from each participant included a blood sample, questionnaire, patient charts and pathology reports.

i. Blood Samples

Fasting blood samples collected at the colonoscopy visit as part of the larger study were used to determine genotype, and for the assessment of biomarker levels of dietary covariates - serum albumin, calcium, folate, triglycerides, cholesterol, lipid ratio (ratio of total cholesterol high density lipoprotein (HDL)) and vitamin B_{12}. All blood samples (buffy coat) were stored prior to analysis in a -20ºC freezer.

ii. Questionnaire and Chart Review

Prior to the colonoscopy visit, participants of the larger study completed a questionnaire to provide information on demographics, lifestyle and dietary factors for CRC. Exposure to dietary HAAs and meat mutagenicity was assessed via the meat consumption module included within the study questionnaire (to be described in detail in Section 6.1.4). Information on height and weight was collected by the research assistant from the patient's chart at the time of colonoscopy.

iii. Pathology Reports

As part of the larger study, copies of pathology reports were received and pathology findings were used for outcome assignment using standard diagnostic criteria (described in detail in section 6.1.5).

6.1.4 Exposure Assessment – Dietary Heterocyclic Aromatic Amines and Meat Mutagenicity

i. Development of the Meat Consumption Module

Phase 2 was conceptualized and planned during the development of the larger study; thus, the dietary questionnaire of the larger study had incorporated a meat consumption module that assessed the frequency of intake in the past 12 months of commonly consumed meat items that

contribute to the majority of dietary HAA exposure according to different cooking methods and preferred doneness level. The development of the meat consumption module for Phase 2 utilized the meat items list from the National Institutes of Health Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease (CHARRED) mutagen database as the starting point for questionnaire development. The CHARRED mutagen database details the specific concentrations of PhIP, MeIQx, DiMeIQx and meat mutagenicity in commonly consumed meat items according to the specific types of cooking method and preferred doneness level. The CHARRED sample questionnaire was adapted for use in Phase 2 and assessed the frequency of intake of meat items including beef steak, hamburger, pork, chicken and fish. Frequency was assessed separately for summer and winter months.

ii. Derivation of Dietary Heterocyclic Aromatic Amine Intakes and Exposure to Meat Mutagenicity

The average intake of specific concentrations of dietary HAAs and meat mutagenicity exposure were estimated by linking the meat consumption module to the CHARRED mutagen database as discussed in Chapter 4 Section 4.1.4. For each participant, the average intake of PhIP, MeIQx, DiMeIQx (expressed in nanograms (ng) per day) and meat mutagenicity exposure (expressed in number of revertant colonies per day) obtained from the CHARRED mutagen database were assigned to responses in the meat consumption module which collected the times per week of each meat item consumed according to the type of cooking method and the usual preferred level of doneness. Unlike Phase 1, usual serving size was not collected in Phase 2; therefore the medium serving size of each meat item within the CHARRED mutagen database was used in all calculations. Total PhIP, MeIQx, DiMeIQx intake and meat mutagencity exposure was summed across the five meat items separately for summer and winter months;

dietary HAA intake and meat mutagenicity exposure in the past year was then averaged across summer and winter months.

As discussed in Chapter 2 Section 2.5.3, meat mutagenicity is a biological measure quantified using the Ames test (1). Meat mutagenicity integrates the mutagenic potential of all the different classes of carcinogens, including HAAs, found in cooked meats. In Phase 2, meat mutagenicity was examined as an exposure of interest to facilitate the investigation of the effect of all dietary HAAs on colorectal adenoma risk acknowledging that other classes of carcinogens also contribute to meat mutagenicity.

6.1.5 Outcome Assessment – Diagnosis of Colorectal Adenoma

As part of the larger study, a copy of pathology reports was received and pathology findings were used for outcome assignment using standard diagnostic criteria by our clinical study coordinator (Dr. Ashbury); the outcome group included patients diagnosed with all histological subtypes of colorectal adenomas including tubulovillous, tubular, serrated and sessile serrated adenomas. Patients with a pathologic diagnosis of hyperplastic polyps (N=28) from the larger study were excluded from Phase 2. Specifically, hyperplastic polyps are considered to have less malignant potential than adenomas and may progress to CRC by means of a different pathway than adenomas (2). Therefore, patients with hyperplastic polyps were excluded from Phase 2 as they warrant a separate analysis from adenomas that is beyond the scope of Phase 2.

6.1.6 Genetic Assessment – Genetic Factors Involved in the Biotransformation of HAAs and the Repair of DNA Adducts and Damage

Effect modification by polymorphisms in genes involved in the biotransformation of HAAs, and the repair of DNA adducts and DNA damage on the relationship between dietary

exposures to HAAs/meat mutagenicity and colorectal adenoma risk were assessed. Genotyping utilizing DNA isolated from blood leukocytes was conducted using the MassARRAY® iPLEX® Gold – SNP Genotyping assay by Genome Quebec in Montréal, Québec. Selection of these genetic factors was discussed in detail in Chapter 3.

6.1.7 Covariate Assessment

Information on personal, lifestyle and dietary factors that were known or suspected risk factors for colorectal adenoma or CRC was collected in the questionnaire with the exception of height and weight which were ascertained on the date of the colonoscopy visit from the patient clinic chart (Appendix 5). The questionnaire of Phase 2 was the same as the one used for Phase 1 when assessing ethnicity, alcohol consumption, smoking status and exposure to environmental tobacco smoke; identical methods were employed for variable conceptualization and thus, are not presented (for details see Section 4.1.7). Biochemical factors were determined in fasting blood samples by Clinical Laboratory Services at Kingston General Hospital, Kingston, Ontario.

i. Characterization of Personal Characteristics

Age and Sex: Age and sex were self-reported by the participants and were assessed through the questionnaire. Specifically, age was considered as both a continuous and categorical variable; categorical representations of age included three categories: less than 50 years, 50 years to less than 60 years and greater than or equal to 60 years.

Body Mass Index (BMI): Weight was abstracted from clinic chart and height was self-reported by the participant at the date of the colonoscopy. BMI was calculated as the ratio of weight to the square of height (kg/m^2). BMI was considered as both a continuous and categorical variable. Categories of BMI were based on the cut points used by the World Health Organization (3). Specially, a BMI of less than 18.50 kg/m^2 was considered 'underweight', between 18.5 and

24.99 kg/m^2 was considered 'normal', between 25.00 and 29.99 was considered 'overweight' and a BMI of greater than 30.00 was considered 'obese'. The 'underweight' (BM<18.50) category was grouped with 'normal' due to small cell size (n=1).

Previous Diagnosis of Irritable Bowel Syndrome: Previous diagnosis of irritable bowel syndrome (IBS) was assessed in the questionnaire with the question that asked whether a participant had been diagnosed with IBS or not.

Family History of CRC: Positive family history of CRC was defined as having a mother, father, sibling or child with a diagnosis of CRC.

ii. Characterization of Lifestyle Factors

Caffeinated Beverage Consumption: Information on average caffeinated beverage (coffee, tea and soft drinks containing caffeine) consumption in the past year was abstracted from the questionnaire. Caffeine consumption was derived and conceptualized based on the method described by Michels et al (4). The caffeine contents for caffeinated coffee, tea and soft drinks were obtained from the U.S. Department of Agriculture food composition sources; 137 mg of caffeine per cup of coffee, 47 mg of caffeine per cup of tea and 46 mg of caffeine per can or bottle of cola beverage were used for calculations. Specific caffeine intake (mg/day) from caffeinated coffee, tea and soft drink consumption was calculated by multiplying the frequency of intake (cups/day) by their specific caffeine content (mg/cup). Average daily caffeine intake was then summed across the caffeine intakes derived from caffeinated coffee, tea and soft drink consumption. Caffeine intake was considered as both a continuous and categorical variable; categorization was conducted according to quartiles of intake among the non-event group.

Physical Activity: Physical activity was assessed through the short-format, self-administered version of the International Physical Activity Questionnaire to quantify a participants' physical

activity in the past year. This questionnaire is comprised of seven questions that collected the number of days per week and the minutes per day during the past year spent engaged in vigorous and moderate activity, and walking in the past year. All vigorous, moderate and walking time variables were truncated to a maximum of 180 minutes to permit a maximum of 21 hours of activity in a week. Energy expenditure in metabolic equivalent of tasks (METs) was then determined for vigorous, moderate and walking activities using the Compendium of Physical Activities; specifically, 3.3 METs was used for walking, 4.0 METs for moderate activity and 8.0 METs for vigorous activity. MET minutes per week for each activity were calculated by multiplying the number of days per week times the minutes per day spent in each activity times the MET value of the activity (5). Total physical activity (in MET-minutes per week) was then calculated based on the summation of the MET-minutes spent in walking, moderate activity and vigorous activity per week. Total physical activity was assessed both as a continuous and a categorical variable and categories of physical activity were based on quartiles among the non-event group.

iii. Characterization of Dietary Factors

Dietary Factors: Dietary factors including protein, total fat, carbohydrate, total energy, and fiber intakes were derived based on the dietary portion of the questionnaire. The dietary portion of the questionnaire was an abbreviated dietary assessment intended to properly rank individuals according to their reported intake of a few specific nutrients (6). The Buffalo Abbreviated Dietary Instrument was modified and adapted for this study; modifications were made to ensure that the instrument reflected the diet of Canadians using the Canadian Food Guide as a reference (7). The questionnaire captured 87 fruits, vegetables, meats, dairy products and grain products. Participant were asked to indicate their average frequency of consumption of each item over the

last 12 months in categories of: never, less than 1 per week, 1 per week, 2 to 3 per week, 4 to 6 per week, once per day and more than once per day. For each food item, the specific protein, total fat, carbohydrate, total energy and fiber value in units per 100 grams of food was extracted from Statistics Canada's Canadian Nutrient File. In the questionnaire, portion sizes were not specified and thus, 100 grams was used as the standard serving size. As well, in the questionnaire, some food items were grouped together (e.g. apples and applesauce) for the assessment of the frequency of intake. To calculate the specific nutrient intakes for these grouped food items the average of each specific nutrient was utilized. Daily intakes of each food in units per day were calculated by multiplying participants' reported frequency of intake (100 grams per day) by the number of units per 100 grams of each specific nutrient. Total dietary protein (g/day), fat (g/day), carbohydrate (g/day), energy (kcal/day) and fiber (g/day) intakes were derived by summing the nutrients from the 87 food items. Dietary factors were considered as both continuous and categorical variables; categorical representations were based on quartiles among the non-event group.

iv. Characterization of Biochemical Factors

Biochemical Factors: Biochemical factors including serum albumin, calcium, triglycerides, cholesterol, lipid ratio, folate and vitamin B_{12} were quantified in the fasting blood samples provided by the participant on the day of colonoscopy. All biochemical factors with the exception of lipid ratio were conceptualized as both a continuous and categorical variable; categorization was based on quartiles among the non-event group.

v. Characterization of Genetic Factors

The polymorphisms under investigation in Phase 2 and their respective genotype categories and functional effects are listed in Table 6.1. Specifically, heterozygotes were

grouped with the homozygote variant category to maximize statistical efficiency. Like Phase 1, the two polymorphisms in CYP1B1*2, rs10012 and rs1056827, are genetically linked and thus, the haplotype of CYP1B1, combining genotypes of rs10012 ‘CG or GG’ with rs105687 ‘GT or TT,’ was examined for all analyses.

Table 6.1. Genetic factors of interest to Phase 2, their genotype categories and associated functional effects

Function	Gene	rs	Genotype Category	Functional Effect	References
Bioactivation	CYP1A2	762551	AA AC/CC	Higher bioactivation Referent	(8)
	CYP1B1	10012	CG/GG CC	Higher bioactivation Referent	(8-11)
	CYP1B1	1056827	GT/TT GG	Higher bioactivation Referent	(9)
	NAT2	1041983	CC CT/TT	Rapid Intermediate/Slow	(12)
DNA Repair	XPA	1800975	CC CT/TT	Lower DNA repair Referent	(13,14)
	XPD	13181	CC/CA AA	Lower DNA Repair Referent	(15,16)
	XPD	1799793	AA/AG GG	Lower DNA Repair Referent	(15-17)
	XRCC1	25487	AA/AG GG	Lower DNA Repair Referent	(18)
	ADPRT	1136410	CC/CT TT	Lower DNA Repair Referent	(19,20)
	XPC	2228001	CC/CA AA	Lower DNA Repair Referent	(16)
	XRCC3	861539	TT/TC CC	Lower DNA Repair Referent	(17)

6.1.8 Data Validity

i. Genetic Data

For the genetic data, measures were undertaken to ensure the inter-assay reliability of the data. In a separate study funded by Colon Cancer Canada, 270 participants in Phase 2 were additionally genotyped for polymorphisms in the ADPRT (rs1136410), XPD (rs13181 and

rs1799793), XPC rs 2228001), XRCC1 (rs25487) and XRCC3 (rs861539) using established methods for genotyping conducted by Queen's Laboratory for Molecular Pathology, Queen's University, Kingston, Ontario. The inter-assay reliability was found to be 100%. In addition, for intra-reliability, repeat assays for 10% of the samples were performed and concordance was found to be 100%. Further evaluation of the genetic data validity compared the obtained genotype distributions to published literature.

6.1.9 Data Management

All consenting participants were assigned sequential four-digit identification (ID) numbers. This ID number was used to identify participants on all data collection forms and samples. Information abstracted from questionnaires was managed using Microsoft© Access. Genetic data, provided by Genome Quebec, was kept in Excel files. Pathology reports of any abnormal tissue removed during the colonoscopy were obtained to assign study outcomes by our clinical study coordinator. All files containing identifying information such as consent forms, patient names, mailing addresses and corresponding subject ID numbers was held apart from the data pertaining to the questionnaires, study outcomes and biologic samples in a separate locked filing cabinet only accessible to study personnel.

6.1.10 Data Analysis

Descriptive statistics were generated for all variables considered in this study. For categorical variables, the frequencies of categories were analyzed and when categorical cell sizes contained less than 5% of the study sample the categories were collapsed. For continuous variables, distribution was described by the mean, standard deviation (SD), median, and 25th and 75th percentiles.

i. Outcome Representation

Logistic regression was utilized as the basic framework for the outcome contrast of event (adenoma) versus non-event (healthy) groups to determine whether exposure to a) dietary HAAs and b) meat mutagenicity differed between patients with adenomas and individuals with no abnormality detected during colonoscopy while controlling for potential confounders.

ii. Exposure Representation

For dietary HAA intakes and meat mutagenicity exposure, *a priori*, three exposure representations were considered: continuous, equal-distant categories and dichotomous representation. First, exposures were conceptualized as a continuous variable to increase statistical efficiency. Second, the use of equal-distant categories was selected as the most appropriate categorical representation given that no natural cut points were obvious and quantile representation would yield categories that may not be meaningful (i.e. due to the skew of the distribution). Specifically, four equal-distant categories were created based on the distribution of the non-event group while ensuring a minimum of 15% of the study population within each category. Using dietary PhIP as an example, the highest category of PhIP was created by categorizing those above the 75th percentile of the study population as Category 4 of PhIP intake (PhIP >348.91 ng/day). Three equal-distant categories were thus created by dividing the 75th percentile cut-off (348.91 ng/day) by three, yielding Category 1 (0 to 116.30 ng/day), Category 2 (116.31 ng/day to 232.61 ng/day) and Category 3 (232.62 ng/day to 348.91 ng/day). Finally, dichotomization of exposures based on the 75th percentile in relation to colorectal adenoma risk was also explored.

iii. Assessment of Confounding

For Phase 2 of this research, the following potential confounders were considered as they have been shown in the literature to be related to the development of colorectal adenomas: sex, age, ethnicity, smoking, environment tobacco smoke, physical activity, BMI, previous diagnosis of IBS, family history of CRC, caffeine consumption, alcohol consumption, dietary intakes of protein, total fat, carbohydrate, energy and fiber, and blood measures of albumin, calcium, folate, triglyceride, cholesterol, lipid ratio and vitamin B_{12}.

In Phase 2, the consideration of variable representation for continuous covariates had the ultimate goal of appropriate control of confounding and was based on two additional priorities: 1) interpretation of effects and 2) linearity of the covariate-outcome relationships. Continuous covariates included age, physical activity, BMI and intake of caffeine, alcohol, protein, fat, carbohydrate, energy and fiber, and blood measures of albumin, calcium, folate, triglycerides, cholesterol, lipid ratio and vitamin B_{12}. First, since covariates were selected based on their established relationship with adenoma risk, comparability with the established literature where the utilization of categorical representations is most common was a priority. All continuous covariates were categorized into quartiles based on the distribution among the non-event population with the exception of age and alcohol consumption which were categorized according to cut points commonly used in the CRC literature (21-22). Second, if a linear relationship was observed between a covariate and adenoma risk, use of continuous representation was prioritized since it would provide an appropriate representation and minimize the number of parameters for model building. Linear relationships were identified by a qualitative evaluation of the dose-response pattern of covariates represented categorically as well as the assessment of linear trend across categories. Trend tests were performed by assigning the median of each category as

scores and computed by adding the continuous variable to the logistic model predicting colorectal adenomas. P-values of less than 0.10 for the trend test with evidence of a linear dose-response relationship upon visual inspection were considered linear and continuous representations were selected, otherwise categorical representations were utilized. For genetic factors, Chi-Square test was used to evaluate the difference between observed and expected genotype frequencies according to the Hardy-Weinberg Equilibrium. Polymorphisms under investigation in Phase 2 may deviate from the Hardy-Weinberg Equilibrium since participants were recruited from an underlying CRC screening cohort that would overly represent those with a family history of CRC.

Correlations between dietary HAAs (PhIP, MeIQx, DiMeIQx) and meat mutagenicity were calculated using Spearman rank correlation. Since dietary HAAs and meat mutagenicity are all estimated from the same dietary measures (frequency of specific meat intake according to different cooking practices and preferred level of doneness) collinearity was anticipated to be an issue and therefore, analysis was conducted in separate models.

Stepwise selection was the main variable selection strategy used to create a parsimonious model of covariates that was predictive of colorectal adenoma risk using a liberal p-value of 0.20. This conventional regression method focuses on the association between a covariate and the outcome of interest and a liberal significance level was used to avoid under-selection of potential covariates (23).

iv. Assessment of Main Effects

The main effects of dietary exposure to a) HAAs and b) meat mutagenicity on colorectal adenoma risk, while controlling for potential confounders, were assessed in separate models using multivariate logistic regression analysis to estimate adjusted odds ratios (OR) and

corresponding 95% confidence intervals (95% CI). Exposures of interest were examined as continuous, equal-distant categories and dichotomous representations. In continuous representations, ORs were interpretable as the odds for colorectal adenoma per SD increase in exposure. It is important to note that since the prevalence of colorectal adenoma in this screening population occurred in greater than 10% of the study population, the adjusted OR would overestimate the prevalence ratio (PR) (24). To overcome this issue, the log-binomial model or a modified Poisson regression model with a robust error variance approach were proposed as alternatives to compute an adjusted PR for a prevalent outcome (25). However, due to the omission of hyperplastic polyps (7.6% of the study population) from our eligible study base, the PR would also not accurately estimate the risk of colorectal adenoma associated with exposure to HAAs and meat mutagenicity. Therefore, multivariate logistic regression was still considered the most appropriate analysis strategy

v. Assessment of Effect Modification

Given the available sample size, interactions between exposure to a) dietary HAAs and b) meat mutagenicity and polymorphisms in genes involved in the biotransformation of HAAs and DNA repair on colorectal adenoma risk were explored since this research had limited statistical power to elucidate an interaction if one existed. Specifically, gene-diet interactions were explored by the inclusion of cross-product terms in the logistic regression models while controlling for confounders. Exposures of interest were represented as dichotomous variables by combining equal-distant Categories 1 and 2, and Categories 3 and 4; this representation was chosen to maximize statistical power.

6.1.11 Sample size and Power

This research investigated the relationships between dietary exposures to a) HAAs and b) meat mutagenicity, and the prevalence of colorectal adenoma in a sample of 342 participants (126 events and 216 non-events). This study had greater than 80% power to detect an OR of 1.95 when comparing the highest to the lowest quartile of exposure. After consideration of the distributions of the exposures of interest, exposures were conceptualized as categorical variables based on equal-distant categories among the non-event group; however, the highest and lowest equal-distant categories yielded similar frequencies to the highest and lowest quartiles and thus the sample size calculation utilizing quartile representations would approximate that of equal-distant categories.

In the analysis of the interactions between exposures to HAAs/meat mutagenicity and genetic susceptibility on the prevalence of colorectal adenoma, exposures were dichotomized based on combining categories 3 and 4 and using category 1 and 2 as the referent category which yields an approximate equal distribution of study subjects. Power calculations for interaction analyses were conducted using the statistical software Quanto (26). Genotypes were collapsed into dichotomous factors according to their models of action. For genotype prevalence of greater than 25% (equivalent to a minor allele frequency of 13%), using a fixed magnitude of $OR_{Exposure}$ of 1.50 and 1.95 (when contrasting categories 3 and 4 against categories 1 and 2) and a range of $OR_{Genetics}$ from 1.25 to 1.50, a sample size of 327 was found to have 80% power to detect an $OR_{Interaction}$ of 3.00 at all genotype frequencies, $OR_{Exposure}$ and $OR_{Genetics}$ ranges (Table 6.2).

Table 6.2. Range of power associated with detectable $OR_{Interaction}$ for a fixed OR_{HAA} and a range of $RR_{Genetics}$.

Genotype Frequency	$OR_{Exposure}$	$OR_{Genetics}$	$OR_{Interaction}$	Power
25%	1.50	1.25	2.50	0.68
		1.25	2.75	0.76
		1.25	3.00	**0.82**
		1.50	2.50	0.68
		1.50	2.75	0.76
		1.50	3.00	**0.82**
	1.95	1.25	2.50	0.67
		1.25	2.75	0.75
		1.25	3.00	**0.81**
		1.50	2.50	0.67
		1.50	2.75	0.75
		1.50	3.00	**0.81**
50%	1.50	1.25	2.50	0.71
		1.25	2.75	0.78
		1.25	3.00	**0.84**
		1.50	2.50	0.69
		1.50	2.75	0.76
		1.50	3.00	**0.82**
	1.95	1.25	2.50	0.70
		1.25	2.75	0.77
		1.25	3.00	**0.82**
		1.50	2.50	0.67
		1.50	2.75	0.75
		1.50	3.00	**0.81**

6.1.12 Additional Analyses

i. Sex-Specific Analysis

A sex-specific analysis of the relationships between a) dietary HAA intake and b) meat mutagenicity exposure on colorectal adenoma risk was undertaken as an additional analysis due to the emerging hypothesis of an inhibiting effect of estrogens on enzymes involved in the biotransformation in HAAs (27,28). Sex-specific analyses were undertaken with the inclusion of product terms into the multivariate logistic regression model.

ii. Use of OR for Prevalent Outcomes

An additional analysis was conducted comparing the OR and PR, and their corresponding 95% CI derived from logistic and a weighted modified Poisson regression model with a robust error variance estimator to evaluate the extent of discordance between OR and PR based on the full study population (N=370). Specifically, hyperplastic polyps were grouped with the non-event group by weighting the non-event group by 1.13 (weighting was determined by adding 28 hyperplastic polyps to 216 non-events and dividing the sum by 216 non-events). The OR and PR associated with having an adenoma among equal-distant representations of exposure (HAAs and meat mutagenicity) were contrasted. In addition, the OR and PR derived from gene-diet interactions between dietary PhIP and polymorphisms in *CYP1B1* and *XPD* on colorectal adenoma risk were also examined.

6.1.13 Sensitivity Analyses

i. Alternative Confounder Selection Approach

Stepwise selection was the main variable selection strategy used to create a parsimonious model of covariates that was predictive of colorectal adenoma risk using a liberal p-value of 0.20. Predictors of colorectal adenoma were included in all multivariable analyses. A sensitivity analysis was conducted to evaluate the impact of the confounder selection approach used by contrasting results obtained using a parsimonious approach versus a change-in-estimate approach.

A change-in-estimate approach to confounder assessment selects covariates based on their effect on the exposure-outcome relationship. First, starting with a full model containing all identified predictors of colorectal adenoma through the parsimonious approach and the exposure of interest (e.g. PhIP), in a stepwise fashion, each confounder was deleted and the covariate that

caused the smallest change in the exposure effect estimate (e.g. OR) upon deletion was removed. The process was repeated and terminated when deletion of each of the remaining variables caused a relative change of more than 5% in the exposure effect estimate when compared to the full model estimate containing all potential confounders (i.e. the starting model) (29). Specifically, the change-in-estimate method was used to identify confounders for PhIP using the equal-distant representation; the OR of Category 4 versus Category 1 of PhIP was used as the parameter estimate of interest and the results of the main effect analyses were compared between those obtained for the parsimonious approach and the change-in-estimate approach. For gene-diet interactions, the effect of the confounder selection approach was assessed via contrasting the results obtained for the interactions between PhIP and polymorphisms in *CYP1B1* and *XPD* among the parsimonious versus change-in-estimate approach. The change-in-estimate procedure utilized the OR in categories 3 and 4 versus categories 1 and 2 of PhIP intake in those with higher bioactivation (CYP1B1 rs10012 'CG or GG' and rs1056827 'GT or TT' genotypes) and lower DNA repair activity (XPD rs13181 CC or CA genotypes and/or XPD rs1799793 'AA or AG' genotypes) as the parameter estimates of interest.

ii. Impact of Adjustment for Total Energy Intake

A sensitivity analysis was conducted to assess the implication of adjusting for total energy intake. In nutritional epidemiology, total energy intake deserves consideration since the level of energy intake may be a primary determinant of the outcome, is likely to be correlated with dietary exposures but may not on the causal pathway between exposure and outcome. Therefore, adjustment for total energy intake in epidemiologic analyses is strongly advocated (30). Results obtained for the main effect analysis of dietary exposure to a) HAAs and b) meat

mutagenicity as well as the gene-diet interactions, on colorectal adenoma risk were compared for multivariable analysis with and without adjustment for total energy intake.

6.2 Ethical Considerations

To ensure that this thesis dissertation complied with ethical standards for research on human subjects, Phase 2 was approved by the Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (Appendix 6). With respect to risks and benefits, informed consent and confidentiality, the following should be noted:

Phase 2:

- The risk associated with blood draw was minimal
- As stated in the consent form, there were no direct benefits to the subjects but this research could benefit future studies in understanding the role of HAAs in colorectal carcinogenesis
- Throughout the study, procedures were in place related to entry and storage of personal and questionnaire data to protect confidentiality and anonymity at all times. Blood and tissue specimens were coded with a non-identifiable subject number assigned at the onset of the study. Pathology reports of any abnormal tissue removed during the colonoscopy were de-identified and reviewed by the clinical study coordinator to assign subjects to the correct outcome group.

6.3 References

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Chapter 7: Phase 2 Results

7.1 Phase 2: Dietary HAA Intake and Risk of Colorectal Adenomas

This dissertation was designed to inform on the relationship between heterocyclic aromatic amines (HAAs) and colorectal adenoma risk in two epidemiologic studies (Phase 1 and 2). The purpose of Phase 2 was to determine whether HAAs and meat mutagenicity play a role in colorectal carcinogenesis through an influence on the risk of developing colorectal adenomas and whether this association is modified by variation in genetic susceptibility to biotransform HAAs and repair DNA. The relationships of interest were investigated in a cross-sectional study of 342 patients undergoing a screening colonoscopy. This Chapter presents the results of Phase 2.

7.1.1 Phase 2 Objectives

Objective #1 investigated whether a relationship existed between: a) dietary exposure to HAAs and the prevalence of colorectal adenomas and; b) meat mutagenicity exposure and colorectal adenoma prevalence. Objective #2 determined whether the relationships between: a) dietary exposure to HAAs and the prevalence of colorectal adenomas and; b) meat mutagenicity exposure and colorectal adenoma prevalence (Objective #1) were modified by variation in genes involved in the biotransformation of HAAs and DNA repair.

7.2 Descriptive Statistics

7.2.1 Exposure Distribution

The distribution of PhIP, MeIQx, DiMeIQx and meat mutagenicity are presented in Figure 7.1, 7.2, 7.3 and 7.4, respectively. Table 7.1 presents the summary statistics including mean, standard deviation (SD), median, 25th and 75th percentiles, skewness and W statistic for each exposure of interest. The distribution of all exposures of interest is right-skewed. For all

exposures of interest, the W statistic obtained from the Shapiro-Wilk test of normality was statistically different from 1 indicating a non-normal distribution.

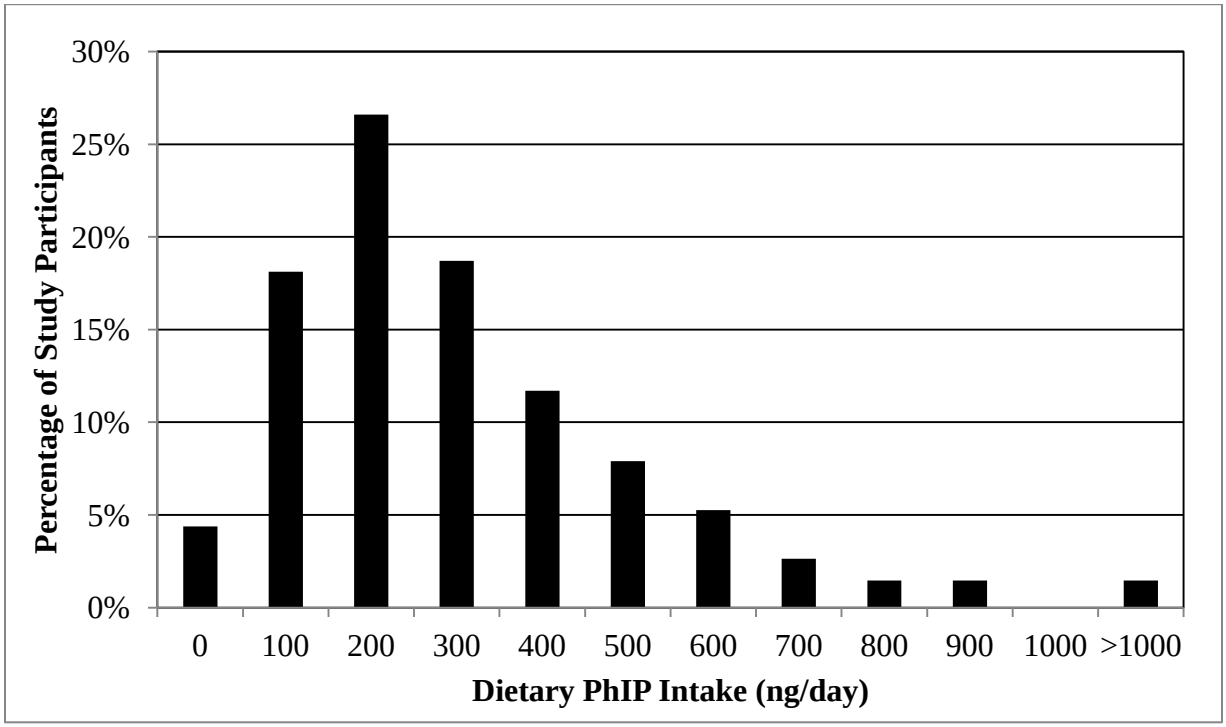


Figure 7.1: Distribution of dietary PhIP in the study population. Midpoint of each interval is indicated.

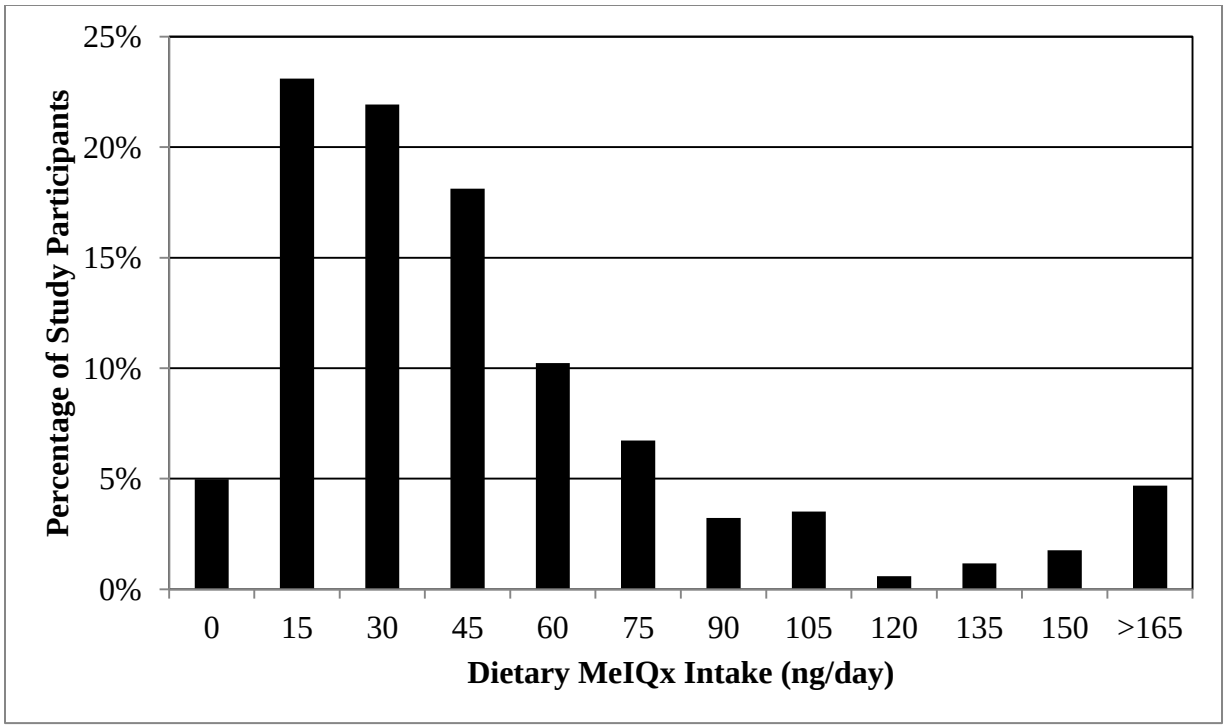


Figure 7.2. Distribution of dietary MeIQx in the study population. Midpoint of each interval is indicated.

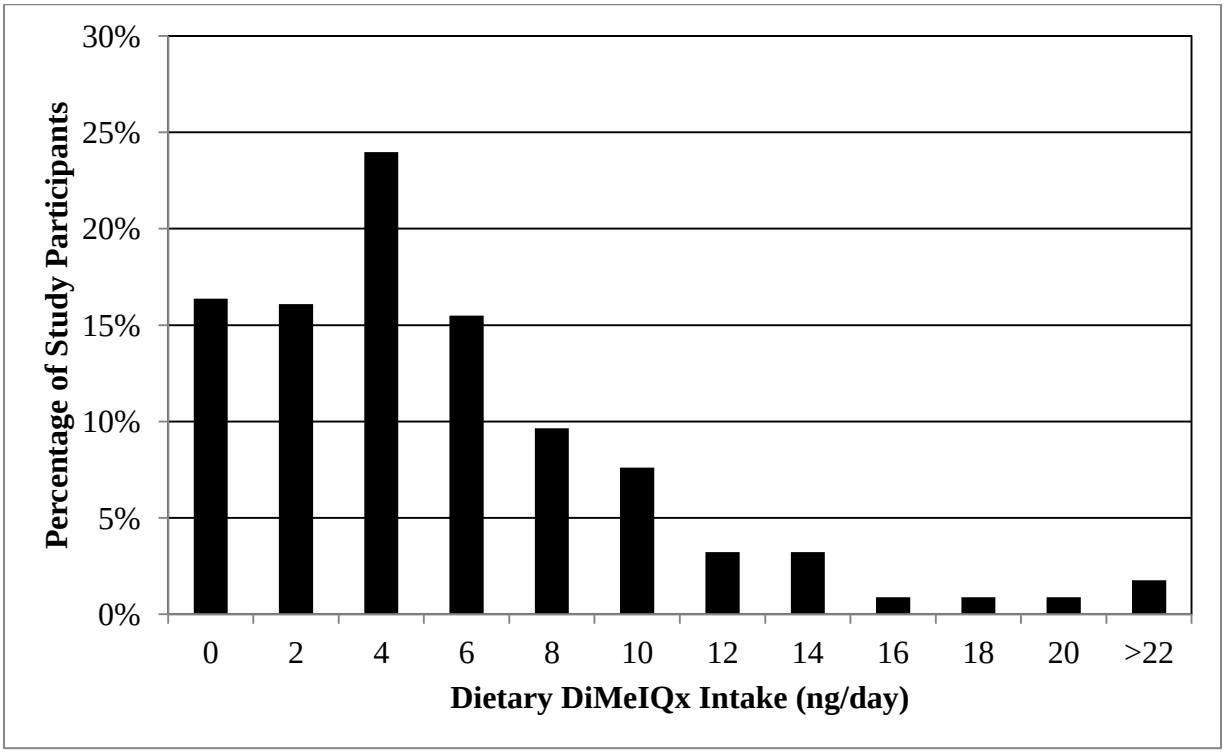


Figure 7.3. Distribution of dietary DiMeIQx in the study population. Midpoint of each interval is indicated.

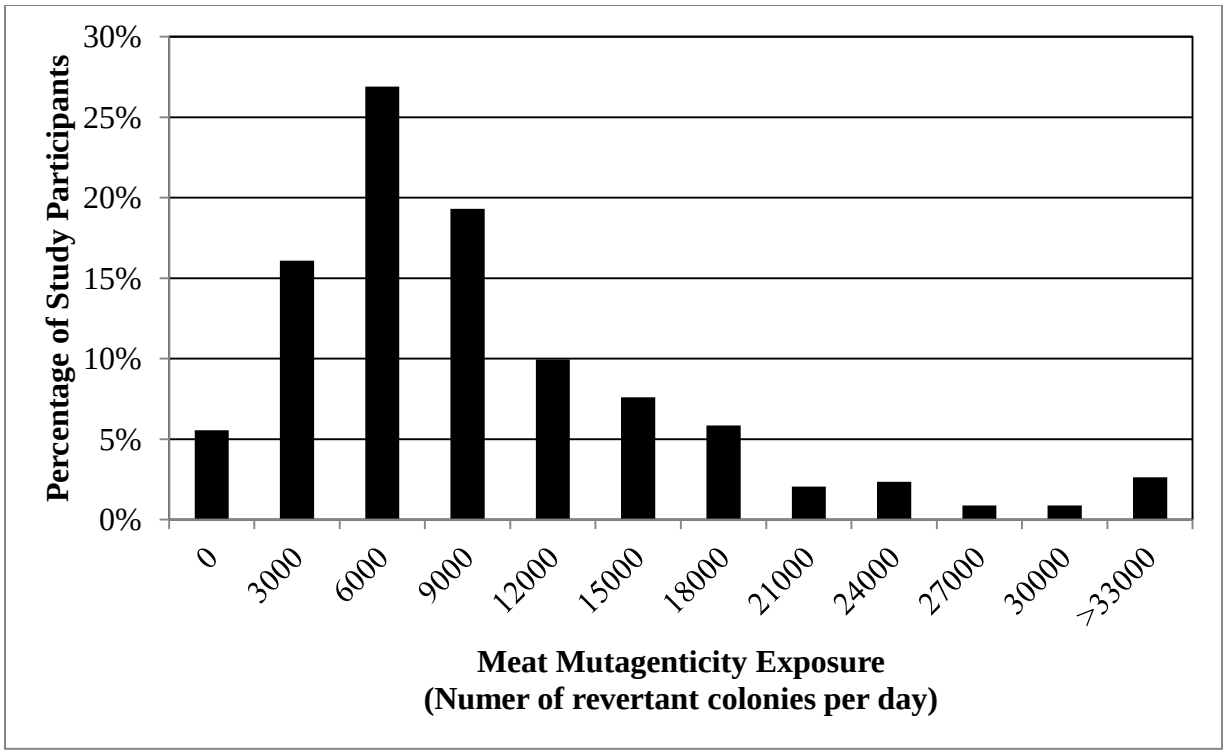


Figure 7.4. Distribution of meat mutagenicity exposure in the study population. Midpoint of each interval is indicated

Table 7.1. Descriptive statistics of exposures of interest in study population

Exposure	Mean	SD	25th Percentile	Median	75th Percentile	Skewness	W
PhIP[1]	271.16	285.94	110.29	201.29	357.44	4.98	0.66
MeIQx[1]	45.91	62.60	12.24	30.26	56.57	4.99	0.59
DiMeIQx[1]	4.76	5.54	1.15	3.49	6.39	3.58	0.71
MeatMutagenicity[2]	8693.42	9470.12	3533.12	6222.89	11151.63	4.30	0.66

[1]Units: ng/day
[2]Units: Number of revertant colonies per day

7.2.2 Outcome Distribution

The frequency and percentages of the colonoscopy findings are presented in Table 7.2. Specifically, 216 (63%) participants undergoing a screening colonoscopy had a normal colonoscopy and 126 (37 %) participants had at least one colorectal adenoma detected. Among those with a diagnosis of colorectal adenoma, tubular adenomas were most prevalent (82%) followed by serrated or sessile serrated (13%) and tubulovillous (6%).

Table 7.2. Distribution of outcome among study population

Outcome	N	Percentage
Normal colonoscopies	216	63.2%
Colorectal Adenoma	126	36.8%
Tubulovillous	7	5.5%
Tubular	103	81.8%
Serrated/Sessile Serrated	16	12.7%

7.2.3 Covariate Distribution

The frequency distribution and percentages for all categorical covariates are presented in Table 7.3. The total sample size of Phase 2 included 342 participants with a greater proportion of females (58%) than males (42%). Overall, the study population was predominately White (94%), never smokers (54%) and never exposed to environmental tobacco smoke (76%). As well, the majority of the study population had a family history of CRC (63%) but no previous diagnosis of irritable bowel syndrome (IBS) (90%). Of the 342 participants, one was missing information on ethnicity and two on environmental tobacco smoke; thus in subsequent analyses including these variables, analyses are conducted on 341 and 340 participants, respectively.

Table 7.3 Descriptive statistics for categorical covariates (N=342)

Covariate	Categories	N	Percent
Sex	Female	198	57.9%
	Male	144	42.1%
Ethnicity	White	320	93.8%
	Other	21	6.2%
	Missing	1	-
Smoking Status	Never	183	53.5%
	Past	115	33.6%
	Current	44	12.9%
Environmental Tobacco Smoke	Unexposed	258	75.9%
	Exposed	82	24.1%
	Missing	2	-
Family History of CRC	Yes	214	62.6%
	No	128	37.4%
Previous Diagnosis of IBS	Previous Diagnosis	36	10.5%
	No Diagnosis	306	89.5%

The descriptive statistics for all continuous variables are presented in Table 7.4. Basic summary statistics including mean, SD, median, 25th and 75th percentiles are reported for each covariate. Of the 342 participants, information was missing for caffeine intake (N=1), physical activity (N=2), body mass index (BMI; N=3), serum albumin (N=6), serum calcium (N=8), serum folate (N=6), serum triglycerides (N=6), serum cholesterol (N=6), lipid ratio (N=6) and serum vitamin B_{12} (N=6); thus in subsequent analyses including these variables, analyses are conducted based on the sample size given in Table 7.4.

Table 7.4. Descriptive statistics for continuous covariates

Covariate	N	Mean	SD	25th Percentile	Median	75th Percentile
Age (years)	342	54.5	5.9	51.0	55.0	59.0
Caffeine Intake (mg/day)	341	254.32	175.82	109.34	250.34	370.00
Alcohol Intake (g/day)	342	12.4	12.7	3.8	10.6	17.4
Physical Activity (MET min/week)	340	4602	3710	1763	3581	6426
Body Mass Index (kg/cm^2)	339	28.4	6.1	24.0	27.7	31.1
Dietary Protein Intake (g/day)	342	62.39	27.98	43.77	58.37	78.36
Dietary Fat Intake (g/day)	342	74.56	40.88	48.61	69.44	93.44
Dietary Carbohydrate Intake (g/day)	342	175.47	87.99	117.00	160.99	218.03
Dietary Energy Intake (kcal/day)	342	1414.87	637.38	998.14	1356.96	1750.71
Dietary Fiber Intake (g/day)	342	26.62	14.82	16.09	24.25	34.19
Fruit and Vegetable Intake (times/day)	342	4.5	2.8	2.8	4.1	5.6
Serum Albumin (g/L)	336	41.7	4.3	40.0	42.0	44.0
Serum Calcium (mmol/L)	334	2.27	0.22	2.24	2.31	2.38
Serum Folate (nmol/L)	336	35.0	9.9	26.9	36.7	45.4
Serum Triglycerides (mmol/L)	336	1.32	0.79	0.76	1.12	1.70
Serum Cholesterol (mmol/L)	336	5.02	1.19	4.18	4.99	5.79
Lipid Ratio	336	4.2	1.4	3.2	3.9	5.0
Serum Vitamin B_{12} (pmol/L)	336	317.8	170.7	210.0	276.0	397.0

7.2.4 Covariate Representation

The selection of a categorical versus continuous representation for continuous covariates was performed by a qualitative assessment of whether the odds ratio (OR) across covariate categories followed a dose-response trend predicting colorectal adenoma risk. Table 7.5 presents the ORs, 95% confidence intervals (95% CI) and p-values obtained from trend tests for all continuous covariates that were categorized into quartiles based on the distribution among the non-events with the exception of age and alcohol consumption. Covariates with an indication of a linear dose response trend were modelled utilizing continuous representations, otherwise categorical representations were utilized. Utilizing this strategy for determination of covariate representation, categorical representations were chosen for age, intake of caffeine, alcohol, total energy, and fruit and vegetables, physical activity, and serum albumin, folate and cholesterol. For BMI, dietary protein, fat, carbohydrate and fiber intakes, and serum calcium, triglycerides and vitamin B_{12} continuous representations were utilized.

Table 7.5. Consideration of covariate representation for continuous covariates utilizing the test of linear trend

Covariate	Categories	N (%)[1]	OR (95% CI)	p[2]	Representation
Age (years)	39-49	61 (17.8)	Referent	0.31	Categorical
	50-59	198 (57.9)	1.32 (0.71, 2.44)		
	≥60	83 (24.3)	1.46 (0.73, 2.93)		
Caffeine Intake (mg/day)	<107.02	77 (22.6)	Referent	0.33	Categorical
	107.03-207.54	80 (23.5)	0.79 (0.40, 1.56)		
	207.55-370.71	100 (29.3)	1.67 (0.90, 3.09)		
	≥370.72	84 (24.6)	1.09 (0.57, 2.09)		
Alcohol Intake (g/day)	Abstainers	54 (15.8)	Referent	0.16	Categorical
	Light	183 (53.5)	1.20 (0.63, 2.30)		
	Moderate	105 (30.7)	1.57 (0.79, 3.14)		
Physical Activity (MET min/week)	≤1865	89 (26.2)	Referent	0.75	Categorical
	1866-3913	89 (26.2)	1.00 (0.55, 1.83)		
	3914-6363	74 (21.8)	0.57 (0.29, 1.11)		
	≥6364	88 (25.9)	0.97 (0.53, 1.78)		
Body Mass Index (kg/m^2)	<18.5-24.9	110 (32.5)	Referent	0.02	Continuous
	25.0-29.9	123 (36.3)	1.63 (0.94, 2.83)		
	≥30.0	106 (31.3)	1.95 (1.11, 3.34)		
Dietary Protein (g/day)	≤45.38	99 (29.0)	Referent	0.06	Continuous
	45.39-61.43	82 (24.0)	0.62 (0.34, 1.14)		
	61.44-81.78	83 (24.3)	0.64 (0.35, 1.17)		
	≥81.79	78 (22.8)	0.53 (0.29, 0.99)		
Dietary Fat (g/day)	≤51.27	92 (26.9)	Referent	0.09	Continuous
	51.28-72.52	91 (26.6)	0.93 (0.52, 1.68)		
	72.53-97.91	83 (24.3)	0.80 (0.44, 1.48)		
	≥97.92	76 (22.2)	0.58 (0.30, 1.11)		
Dietary Carbohydrate (g/day)	≤119.48	92 (26.9)	Referent	0.09	Continuous
	119.49-169.00	87 (25.4)	0.89 (0.48, 1.58)		
	169.01-229.68	88 (25.7)	0.90 (0.49, 1.63)		
	≥229.69	75 (21.9)	0.55 (0.29, 1.06)		
Dietary Energy (kcal/day)	≤1017.02	92 (26.9)	Referent	0.18	Categorical
	1017.03-1388.49	87 (25.4)	0.87 (0.48, 1.58)		
	1388.50-1781.56	84 (25.6)	0.79 (0.43, 1.45)		
	≥1781.57	79 (23.1)	0.66 (0.35, 1.24)		

Dietary Fiber (g/day)	≤16.42	90 (26.3)	Referent	0.06	Continuous
	16.43-25.40	91 (26.6)	1.03 (0.57, 1.86)		
	25.41-35.94	88 (25.7)	0.94 (0.52, 1.72)		
	≥35.95	73 (21.4)	0.53 (0.27, 1.03)		
Fruit and Vegetable Intake (servings/day)	≤2.9	88 (25.7)	Referent	0.43	Categorical
	3.0-4.1	80 (23.4)	0.80 (0.43, 1.52)		
	4.2-6.0	99 (29.0)	1.45 (0.81, 2.60)		
	≥6.1	75 (21.9)	0.65 (0.33, 1.26)		
Serum Calcium (mmol/L)	≤2.24	89 (26.6)	Referent	0.07	Continuous
	2.25-2.30	75 (22.5)	0.87 (0.45, 1.68)		
	2.31-2.38	88 (26.3)	0.97 (0.52, 1.81)		
	≥2.39	82 (24.6)	1.87 (1.01, 3.47)		
Serum Albumin (g/L)	≤40.0	91 (27.1)	Referent	0.12	Categorical
	40.1-42.0	81 (24.1)	0.50 (0.26, 0.99)		
	42.1-44.0	87 (25.9)	1.18 (0.65, 2.17)		
	≥44.1	77 (22.9)	1.63 (0.88, 3.02)		
Serum Folate (nmol/L)	≤26.6	83 (24.7)	Referent	0.48	Categorical
	26.7-37.3	92 (28.4)	1.51 (0.82, 2.79)		
	37.4-45.3	61 (18.1)	1.78 (0.90, 3.51)		
	≥45.4	100 (29.8)	0.69 (0.37, 1.31)		
Serum Triglycerides (mmol/L)	≤0.74	75 (22.3)	Referent	0.02	Continuous
	0.75-1.07	76 (22.6)	0.98 (0.49, 1.96)		
	1.08-1.55	86 (25.6)	1.34 (0.69, 2.59)		
	≥1.56	99 (29.5)	1.88 (1.00, 3.54)		
Serum Cholesterol (mmol/L)	≤4.24	89 (26.5)	Referent	0.75	Categorical
	4.25-4.96	74 (22.0)	0.58 (0.30, 1.13)		
	4.97-5.75	84 (25.0)	0.82 (0.44, 1.51)		
	≥5.76	89 (26.5)	1.00 (0.55, 1.82)		
Serum Vitamin B_{12} (pmol/L)	≤212.0	88 (26.2)	Referent	0.02	Continuous
	212.0-287.5	95 (28.3)	1.20 (0.67, 2.16)		
	287.6-419.0	83 (24.7)	0.81 (0.44, 1.51)		
	≥419.1	70 (20.8)	0.49 (0.24, 0.97)		

[1]Percentages are calculated based on the N of each covariate; missing information on caffeine intake (N=1), physical activity (N=2), serum albumin (N=6), serum calcium (N=8), serum folate (N=6), serum triglycerides (N=6), serum cholesterol (N=6), lipid ratio (N=6) and serum vitamin B_{12} (N=6)

[2]p-value obtained from a trend test

7.2.5 Genetic Factors

The frequency distribution and percentages for all genetic factors are presented in Table 7.6. The total sample size available for the genetic analysis included 327 participants; though missing genotype information was observed for CYP1A2 (N=14), CYP1B1 (rs10012: N=15; rs1056827 N=1), NAT2 (N=2), ADPRT (N=1), XPC (N=2), XRCC1 (N=10) and XRCC3 (N=10). The genotype distributions were all consistent with published literature among Caucasians (1-12). All polymorphisms with the exception of CYP1A2 (rs762551) and XPC (rs2228001) were in accordance with the Hardy-Weinberg Equilibrium distributions ($p>0.05$). Discordance between the observed and expected distributions may be a characteristic of the underlying screening cohort from which participants of Phase 2 were recruited.

Table 7.6. Frequency of genetic factors of interest and Chi-Square test of Hardy-Weinberg Equilibrium distributions among total study population

Gene	rs	Genotype Category	N	Percentage	p[1]
CYP1A2	762551	AA	163	50.2%	0.02
		AC	146	44.9%	
		CC	16	4.9%	
CYP1B1	10012	CC	173	55.3%	0.05
		CG	110	35.1%	
		GG	30	9.6%	
CYP1B1	1056827	GG	161	51.6%	0.05
		GT	116	37.2%	
		TT	35	11.2%	
NAT2	1041983	CC	166	50.9%	0.54
		CT	130	39.9%	
		TT	30	9.2%	
XPA	1800975	GG	156	49.2%	0.05
		AG	122	38.5%	
		AA	39	12.3%	
XPD	13181	CC	46	14.1%	0.85
		CA	155	47.5%	
		AA	125	38.3%	
XPD	1799793	AA	36	10.9%	0.62
		GA	152	46.1%	
		GG	142	43.0%	
XRCC1	25487	GG	143	45.1%	0.19
		GA	132	41.6%	
		AA	42	13.2%	
ADPRT	1136410	CC	8	2.5%	0.41
		CT	99	30.4%	
		TT	219	67.2%	
XPC	2228001	AA	117	36.0%	0.02
		CA	173	53.2%	
		CC	35	10.8%	
XRCC3	861539	TT	50	15.4%	0.59
		TC	146	44.9%	
		CC	121	37.2%	

[1]p-value obtained from χ^2 test

7.3 Covariate Associations with Colorectal Adenoma

The distributions of categorical covariates among those with and without colorectal adenomas were compared using the Chi-Square test (Table 7.7). Statistically significant relationships defined at a 0.05 level of significance were observed for sex, smoking status, previous diagnosis of IBS and serum albumin and folate. Specifically, a greater number of males, current and past smokers and those with no previous diagnosis of IBS were observed among the event versus non-event group. For serum albumin and folate, the trends in the difference across quartiles between event and non-event groups were difficult to interpret due to non-linear trends.

Table 7.7. Frequency of categorical covariates among colorectal adenoma events and non-events

Covariate	Categories	$N_{Non\text{-}event}$ (%)[1]	N_{Event} (%)	OR (95% CI)	p[2]
Sex	Female	151 (69.9)	47 (37.3)	Referent	<0.01
	Male	65 (30.1)	79 (62.7)	3.91 (2.46, 6.21)	
Age	39-50	42 (19.4)	19 (15.1)	Referent	0.56
	50-60	124 (57.4)	74 (58.7)	1.32 (0.71, 2.44)	
	60+	50 (23.2)	33 (26.2)	1.46 (0.73, 2.93)	
Ethnicity	White	202 (94.9)	118 (93.7)	Referent	0.91
	Other	13 (6.1)	8 (6.3)	1.05 (0.42, 2.62)	
Smoking Status	Never	130 (60.2)	53 (42.1)	Referent	<0.01
	Past	65 (30.1)	50 (39.7)	1.89 (1.16, 3.07)	
	Current	21 (9.7)	23 (18.2)	2.69 (1.37, 5.26)	
Environmental Tobacco Smoke	Unexposed	166 (77.2)	92 (73.6)	Referent	0.45
	Exposed	49 (22.8)	33 (26.4)	1.22 (0.73, 2.02)	
Family History of CRC	Yes	142 (65.7)	72 (57.1)	Referent	0.11
	No	74 (34.3)	54 (42.9)	1.44 (0.92, 2.26)	
Previous Diagnosis of IBS	Previous Diagnosis	29 (13.4)	7 (5.6)	Referent	0.02
	No Diagnosis	187 (86.6)	119 (94.4)	2.64 (1.12, 6.21)	

Physical Activity (MET min/week)	≤1865	54 (25.0)	35 (28.2)	Referent	0.30
	1866-3913	54 (25.0)	35 (28.2)	1.00 (0.55, 1.83)	
	3914-6363	54 (25.0)	20 (16.2)	0.57 (0.29, 1.12)	
	≥6364	54 (25.0)	34 (26.4)	0.97 (0.53, 1.78)	
Caffeine Intake (mg/day)	<107.02	51 (23.6)	26 (20.8)	Referent	0.10
	107.03-207.54	57 (26.4)	23 (18.4)	0.79 (0.40, 1.56)	
	207.55-370.71	54 (25.0)	46 (36.8)	1.67 (0.90, 3.09)	
	≥370.72	54 (25.0)	30 (24.0)	1.09 (0.57, 2.09)	
Alcohol Intake (g/day)	Abstainers	37 (17.1)	17 (13.5)	Referent	0.38
	Low	118 (54.6)	65 (51.6)	1.20 (0.63, 2.30)	
	Moderate	61 (28.3)	44 (34.9)	1.57 (0.79, 3.14)	
Dietary Energy Intake (kcal/day)	≤1017.02	54 (25.0)	38 (30.2)	Referent	0.62
	1017.03-1388.49	54 (25.0)	33 (26.2)	0.87 (0.48, 1.58)	
	1388.50-1781.56	54 (25.0)	30 (23.8)	0.79 (0.43, 1.45)	
	≥1781.57	54 (25.0)	25 (19.8)	0.66 (0.35, 1.24)	
Fruit and Vegetable Intake (servings per day)	≤2.9	55 (25.5)	33 (26.2)	Referent	0.07
	3.0-4.1	54 (25.0)	26 (20.6)	0.80 (0.43, 1.52)	
	4.2-6.0	53 (24.5)	46 (36.5)	1.45 (0.81, 2.60)	
	≥6.1	54 (25.0)	21 (16.7)	0.65 (0.33, 1.26)	
Serum Albumin (g/L)	≤40.0	58 (27.2)	33 (26.8)	Referent	<0.01
	40.1-42.0	63 (29.6)	18 (14.6)	0.50 (0.26, 0.99)	
	42.1-44.0	52 (24.4)	35 (28.5)	1.18 (0.65, 2.17)	
	≥44.1	40 (18.8)	37 (30.1)	1.63 (0.88, 3.02)	
Serum Folate (nmol/L)	≤26.6	55 (25.8)	28 (22.8)	Referent	0.02
	26.7-37.3	52 (24.4)	40 (32.5)	1.51 (0.82, 2.79)	
	37.4-45.3	32 (15.0)	29 (23.6)	1.78 (0.90, 3.51)	
	≥45.4	74 (34.7)	26 (21.1)	0.69 (0.37, 1.31)	
Serum Cholesterol (mmol/L)	≤4.24	53 (24.9)	36 (29.3)	Referent	0.35
	4.25-4.96	53 (24.9)	21 (17.0)	0.58 (0.30, 1.13)	
	4.97-5.75	54 (25.3)	30 (24.4)	0.82 (0.44, 1.51)	
	≥5.76	53 (24.9)	36 (29.3)	1.00 (0.55, 1.82)	

[1]Percentages are calculated based on the N of each covariate; missing information on caffeine intake (N=1), physical activity (N=2), serum albumin (N=6), serum folate (N=6) and serum cholesterol (N=6)
[2]p-value obtained from a χ^2 test

For continuous variables, differences in means among non-event and event groups were contrasted using t-tests (Table 7.8). Statistically significant relationships defined at a 0.05 level

of significance were observed for dietary intakes of protein and fiber, and lipid ratio, triglycerides and vitamin B_{12}. Specifically, events had lower intakes of protein and fiber than non-events. Biomarker levels of lipid ratio, triglycerides and vitamin B_{12} also differed between non-event and event groups where higher levels of lipid ratio and triglycerides and lower concentration of serum vitamin B_{12} were observed among events versus non-events groups.

Table 7.8. Mean and standard deviation of continuous covariates among colorectal adenoma events and non-events

Covariates	**$N_{Non\text{-}event}$ N=216[1]**		**N_{Event} N=126[1]**		**p[2]**
	Mean	**SD**	**Mean**	**SD**	
Body Mass Index (kg/cm^2)	27.9	6.2	29.2	5.9	0.07
Dietary Protein Intake (g/day)	64.97	29.49	57.98	24.68	0.03
Dietary Fat Intake (g/day)	77.57	43.18	69.40	36.20	0.07
Dietary Carbohydrate Intake (g/day)	181.80	94.75	164.60	74.10	0.08
Dietary Fiber Intake (g/day)	27.95	16.10	24.4	12.0	0.03
Lipid Ratio	4.0	1.3	4.5	1.5	<0.01
Serum Calcium (mmol/L)	2.26	0.22	2.29	0.21	0.18
Serum Triglycerides (mmol/L)	1.23	0.71	1.47	0.89	<0.01
Serum Vitamin B_{12} (pmol/L)	337.6	184.7	283.5	137.4	<0.01

[1] Missing information for BMI (N=3), lipid ratio (N=6), serum calcium (N=8), serum triglycerides (N=6) and serum vitamin B_{12} (N=6)
[2]p-value obtained from a t-test

The distributions of genetic factors among those with and without colorectal adenomas were compared using the Chi-Square test (Table 7.9). All heterozygotes were categorized with the homozygous variants as described in Chapter 5.1.7. Statistically significant relationships defined at a 0.05 level of significance were observed for polymorphisms in the CYP1B1 (rs10012 and rs1056827) and XPC (rs2228001) genes.

Table 7.9. Frequency of genetic factors among colorectal adenoma events and non-events

Gene	rs	Genotype Category	$N_{Non\text{-}event}$[1] (%)	N_{Event}[1] (%)	p[2]
CYP1A2	762551	AA	108 (52.2)	54 (45.8)	0.27
		AC/CC	99 (47.8)	64 (54.2)	
CYP1B1	10012/1056827	CG/GG+GT/TT	79 (41.1)	59 (55.1)	0.02
		CC+GG	113 (58.9)	48 (44.9)	
NAT2	1041983	CC/CT	99 (47.6)	61 (51.7)	0.48
		TT	109 (52.4)	57 (48.3)	
XPA	1800975	CC	101 (49.7)	55 (48.2)	0.80
		CT/TT	102 (50.3)	59 (51.8)	
XPD	13181	CC/CA	123 (59.1)	78 (66.1)	0.21
		AA	85 (40.9)	40 (33.9)	
XPD	1799793	AA/GA	121 (57.9)	65 (55.1)	0.62
		GG	88 (42.1)	53 (44.9)	
XRCC1	25487	AA/AG	116 (57.1)	58 (50.9)	0.28
		GG	87 (42.9)	56 (49.1)	
ADPRT	1136410	CC/CT	67 (32.2)	40 (33.9)	0.76
		CC	141 (67.8)	78 (66.1)	
XPC	2228001	CC/CA	124 (59.6)	84 (71.8)	0.03
		AA	84 (40.4)	33 (28.2)	
XRCC3	861539	TT/TC	129 (63.5)	67 (58.8)	0.40
		CC	74 (36.5)	47 (41.2)	

[1]Percentages are calculated based on the N of each genetic factor; missing information for CYP1A2 (N=14), CYP1B1 (rs10012: N=15; rs1056827 N=1), NAT2 (N=2), ADPRT (N=1), XPC (N=2), XRCC1 (N=10) and XRCC3 (N=10)
[2]p-value obtained from a χ^2 test

7.4 Covariate Associations with Dietary Exposures to Heterocyclic Aromatic Amine and Meat Mutagenicity

The relationships between: a) dietary exposure to HAAs and categorical covariates and; b) meat mutagenicity and categorical covariates, were examined to provide an understanding of their relationships and inform on the potential for confounding. The relationships between

exposures of interest and categorical covariates were examined and tested by the Chi-Square test and Fisher's exact tests to determine whether the distribution of exposures of interest differed according to covariates (Appendix 7: Tables 1-4). Statistically significant relationships defined at a 0.05 level of significance were observed between dietary PhIP and intake of fruit and vegetables. For MeIQx, associations with smoking status, and exposure to environmental tobacco smoke were observed. Ethnicity and smoking status were associated with DiMeIQx; for meat mutagenicity, association with age was observed.

For continuous covariates, differences in means by dichotomous representations of PhIP, MeIQx, DiMeIQx and meat mutagenicity based on combining equal-distant Categories 1 and 2, and Categories 3 and 4 were calculated using t-tests (Appendix 7: Tables 5-8). Statistically significant relationships defined at a 0.05 level of significance were observed for all HAAs, and BMI and lipid ratio. Associations were observed between meat mutagenicity and BMI, dietary protein intake and lipid ratio.

The Spearman correlation coefficients for dietary HAA (PhIP, MeIQx and DiMeIQx) intake and exposure to meat mutagenicity are presented in Appendix 7 Table 9; PhIP, MeIQx, DiMeIQx and meat mutagenicity were found to be highly correlated with each other ($p<0.01$) and were thus modeled in separate analyses.

7.5 Assessment of Confounding

Stepwise selection was used to create a parsimonious model of covariates that were predictive of colorectal adenoma risk using a liberal p-value of 0.20. Sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio were found to be predictors of colorectal adenoma

at a 0.20 level of significance. The ORs and corresponding 95% CIs for identified predictors of colorectal adenomas are presented in Table 7.10.

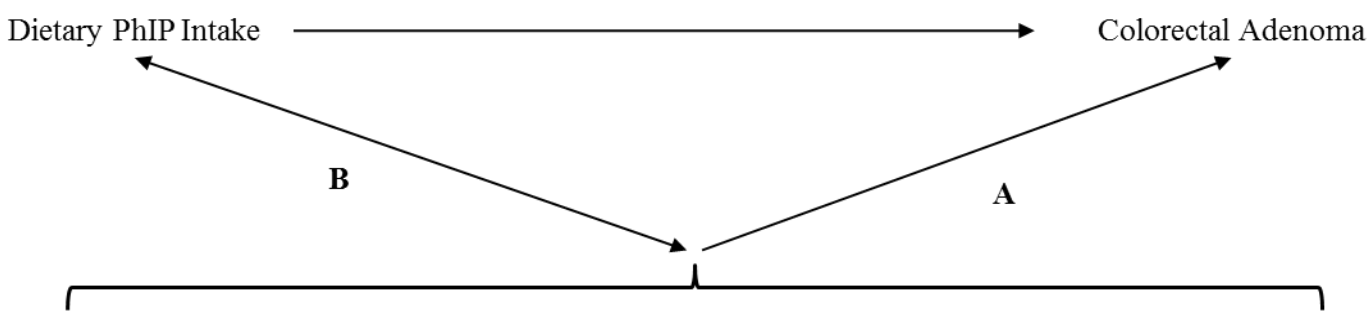
Male sex, being a current or past smoker and higher intakes of fruit and vegetables were predictive of colorectal adenoma risk at 0.05 level of significance. For serum folate, the trends were less interpretable; in general, a higher level of serum folate as represented by quartile 3 compared with quartile 1 was associated with an increase risk of colorectal adenoma. For serum albumin, increasing biomarker levels were associated with a higher risk for colorectal adenomas. For dietary protein intake, increasing intake was associated with a protective effect on colorectal adenoma risk.

Figures 7.5 to 7.7 summarize the extent to which identified predictors of colorectal adenoma may confound the relationship between dietary HAAs and colorectal adenoma risk; Figure 7.8 illustrates the potential for predictors of colorectal adenoma to confound the relationship between exposure to meat mutagenicity and the risk of developing colorectal adenomas. All Figures 7.5 to 7.8 include: 1) directionality of the relationship between each predictor and colorectal adenoma risk and; 2) the bivariate association between each predictor and the exposure of interest. Predictors of colorectal adenoma that were also bivariately associated with dietary HAAs or meat mutagenicity at the 0.20 level of significance are indicated in shaded rows.

Table 7.10. Mutually adjusted OR and 95% CI for predictors of colorectal adenoma risk (N=336)

Covariate	Categories	N Non-events	N Events	Adjusted OR (95% CI)	p[1]
Sex	Female	151	47	Referent	<0.01
	Male	65	79	3.28 (1.89, 5.68)	
Smoking Status	Never	130	53	Referent	<0.01
	Past	65	50	2.08 (1.18, 3.66)	
	Current	21	23	3.59 (1.56, 8.26)	
Previous Diagnosis of IBS	Diagnosis	187	119	Referent	0.17
	No Diagnosis	29	7	2.00 (0.75, 5.33)	
Fruit and Vegetable Intake (servings/day)	<2.9	55	33	Referent	0.02
	2.9-4.1	54	26	1.06 (0.50, 2.24)	
	4.1-6.0	53	46	2.69 (1.29, 5.59)	
	≥6.0	54	21	2.19 (0.86, 5.56)	
Serum Folate (nmol/L)	<26.6	55	28	Referent	0.03
	26.60-37.3	52	40	1.94 (0.94, 4.01)	
	37.3-45.4	32	29	2.96 (1.30, 6.73)	
	≥45.4	74	26	1.23 (0.54, 2.81)	
Serum Albumin (g/L)	<40.0	58	33	Referent	<0.01
	40.0-42.0	63	18	0.41 (0.19, 0.88)	
	42.0-44.0	52	35	1.32 (0.65, 2.67)	
	≥44.0	40	37	1.41 (0.69, 2.88)	
Dietary Protein Intake (g/day)	per s.d.	-	-	0.61 (0.42, 0.87)	<0.01
Serum Vitamin B_{12} (pmol/L)	per s.d.	-	-	0.77 (0.54, 1.10)	0.16
Lipid Ratio	per s.d.	-	-	1.20 (0.94, 1.54)	0.15

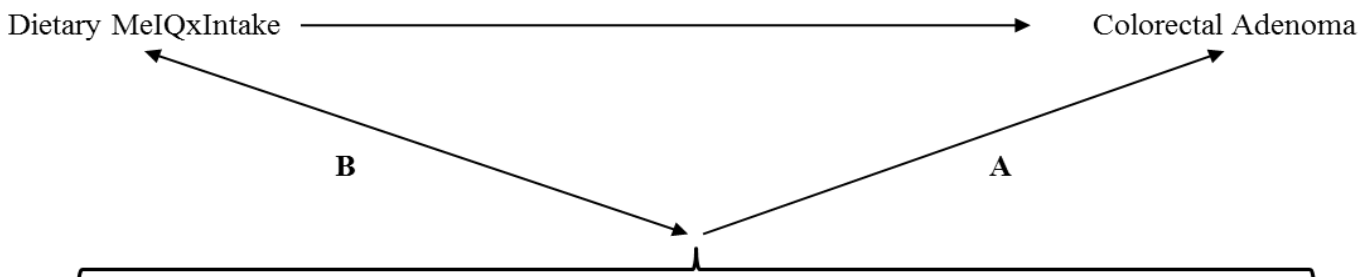
[1]p-value obtained from a Wald Chi-Square Statistic



Predictors of Colorectal Adenoma	A: Higher Risk of Colorectal Adenoma[1]	B: Higher Intake of Dietary PhIP[2]
Sex	Male	No Association
Previous Diagnosis of IBS	No diagnosis of IBS	No Association
Serum Folate	Higher levels	No Association
Serum Vitamin B_{12}	Lower levels	No Association
Dietary Protein	Lower intake	Higher Intake
Serum Lipid Ratio	Higher levels	Higher levels
Smoking Status	Current and Past smokers	Past Smokers
Fruit and Vegetable Intake	Higher intake	Higher intake
Serum Albumin	Higher levels	Higher levels

[1]Directionality of relationship between predictors and colorectal adenoma is based on mutually adjusted OR
[2]Directionality of relationship between predictors and dietary PhIP is based on bivariate associations; an association is defined by $p<0.20$ level of significance

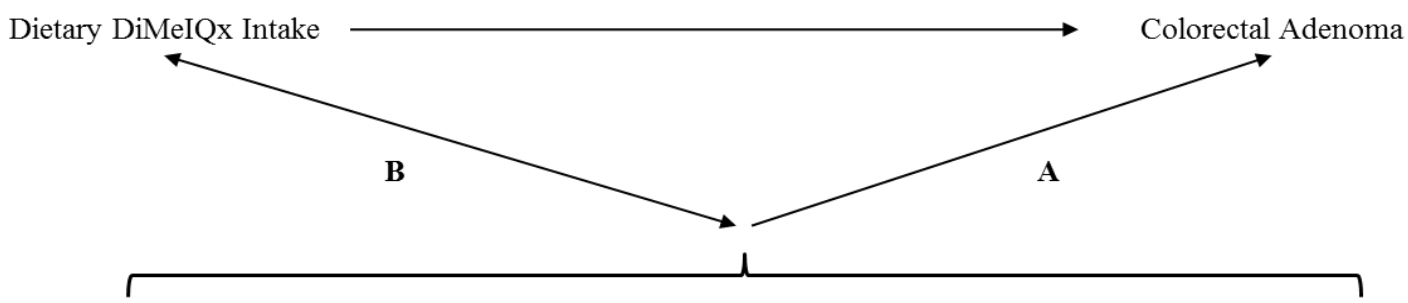
Figure 7.5: Evaluation of the extent of to which predictors of colorectal adenoma may confound the relationship between dietary PhIP and colorectal adenoma risk



Predictors of Colorectal Adenoma	A: Higher Risk of Colorectal Adenoma[1]	B: Higher Intake of Dietary MeIQx[2]
Sex	Male	No Association
Previous Diagnosis of IBS	No diagnosis of IBS	No Association
Serum Folate	Higher levels	No Association
Serum Vitamin B_{12}	Lower levels	No Association
Dietary Protein	Lower intake	No Association
Fruit and Vegetable Intake	Higher intake	No Association
Serum Albumin	Higher levels	No Association
Serum Lipid Ratio	Higher levels	Higher levels
Smoking Status	Current and Past smokers	Past Smokers

[1]Directionality of relationship between predictors and colorectal adenoma is based on mutually adjusted OR
[2]Directionality of relationship between predictors and dietary MeIQx is based on bivariate associations; an association is defined by $p<0.20$ level of significance

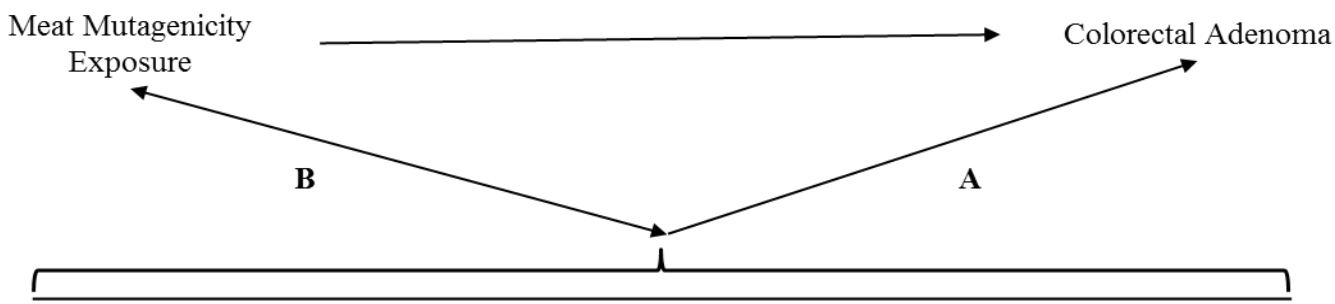
Figure 7.6: Evaluation of the extent of to which predictors of colorectal adenoma may confound the relationship between dietary MeIQx and colorectal adenoma risk



Predictors of Colorectal Adenoma	A: Higher Risk of Colorectal Adenoma[1]	B: Higher Intake of Dietary DiMeIQx[2]
Sex	Male	No Association
Previous Diagnosis of IBS	No diagnosis of IBS	No Association
Serum Folate	Higher levels	No Association
Dietary Protein	Lower intake	No Association
Fruit and Vegetable Intake	Higher intake	No Association
Serum Albumin	Higher levels	No Association
Serum Vitamin B_{12}	Lower levels	Higher levels
Serum Lipid Ratio	Higher levels	Higher levels
Smoking Status	Current and Past smokers	Past Smokers

[1]Directionality of relationship between predictors and colorectal adenoma is based on mutually adjusted OR
[2]Directionality of relationship between predictors and dietary DiMeIQx is based on bivariate associations; an association is defined by $p<0.20$ level of significance

Figure 7.7: Evaluation of the extent of to which predictors of colorectal adenoma may confound the relationship between dietary DiMeIQx and colorectal adenoma risk



Predictors of Colorectal Adenoma	A: Higher Risk of Colorectal Adenoma[1]	B: Higher Intake of Dietary Meat Mutagenicity[2]
Previous Diagnosis of IBS	No diagnosis of IBS	No Association
Serum Folate	Higher levels	No Association
Dietary Protein	Lower intake	No Association
Smoking Status	Current and Past smokers	No Association
Serum Albumin	Higher levels	No Association
Sex	Male	Male
Serum Vitamin B_{12}	Lower levels	Higher levels
Serum Lipid Ratio	Higher levels	Higher levels
Fruit and Vegetable Intake	Higher intake	Higher intake

[1]Directionality of relationship between predictors and colorectal adenoma is based on mutually adjusted OR
[2]Directionality of relationship between predictors and dietary meat mutagenicity is based on bivariate associations; an association is defined by $p<0.20$ level of significance

Figure 7.8: Evaluation of the extent of to which predictors of colorectal adenoma may confound the relationship between meat mutagenicity exposure and colorectal adenoma risk

7.6 Main Analysis

The multivariable relationships between: a) dietary HAAs; and b) exposure to meat mutagenicity, on colorectal adenoma risk were examined using multiple logistic regression among 336 participants with complete data. Exposure conceptualization included continuous and categorical representations via equal-distant categories and dichotomization based on the 75th percentile. The main effects of PhIP, MeIQx, DiMeIQx and meat mutagenicity were individually assessed in separate models while controlling for predictors of colorectal adenomas. Table 7.11 presents the results for continuous representations and Table 7.12 and 7.13 presents the results for categorical representations via equal-distant categories and dichotomization based on the 75th percentile, respectively. In both crude and multivariable analyses, none of the representations of dietary HAAs were significantly associated with colorectal adenoma risk.

Table 7.11. Multivariable analysis of colorectal adenoma risk associated with dietary HAA intake (per SD) and exposure to meat mutagenicity (N=336)

Exposure	Crude OR (95% CI)	p[1]	Adjusted OR[2] (95% CI)	p[1]
PhIP (per SD)	1.05 (0.84, 1.32)	0.65	1.06 (0.82, 1.38)	0.66
MeIQx (per SD)	1.14 (0.96, 1.36)	0.14	1.16 (0.94, 1.43)	0.16
DiMeIQx (per SD)	1.11 (0.92, 1.33)	0.27	1.13 (0.92, 1.39)	0.26
Meat Mutagenicity (per SD)	1.11 (0.92, 1.34)	0.29	1.13 (0.91, 1.41)	0.27

[1]p-value obtained from a Wald Chi-Square Statistic
[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio

Table 7.12. Multivariable analysis of colorectal adenoma risk associated with dietary HAA intake and exposure to meat mutagenicity as represented by a 4-level equal-distant categories

	Categories (ng/day)	% Non-events (N=213)	% Events (N=123)	Crude OR (95% CI)	p[1]	Adjusted OR[2] (95% CI)	p[1]
PhIP	Category 1: ≤116.30	25.9	29.4	Referent	0.58	Referent	0.65
	Category 2: 116.31-232.61	31.0	26.2	0.75 (0.41, 1.34)		0.77 (0.38, 1.55)	
	Category 3: 232.62-348.91	18.1	15.1	0.74 (0.37, 1.47)		0.66 (0.29, 1.51)	
	Category 4: ≥348.92	25.0	29.4	1.04 (0.58, 1.87)		1.03 (0.50, 2.11)	
MeIQx	Category 1: ≤18.54	35.7	27.0	Referent	0.22	Referent	0.29
	Category 2: 18.55-37.09	25.9	26.2	1.34 (0.74, 2.41)		1.37 (0.67, 2.83)	
	Category 3: 37.10-55.63	13.4	20.6	2.03 (1.04, 3.95)		2.19 (0.99, 4.81)	
	Category 4: ≥55.64	25.0	26.2	1.38 (0.77, 2.50)		1.37 (0.99, 4.81)	
DiMeIQx	Category 1: ≤2.06	33.8	31.0	Referent	0.62	Referent	0.68
	Category 2: 2.07-4.12	27.8	23.8	0.94 (0.52, 1.68)		0.78 (0.38, 1.61)	
	Category 3: 4.13-6.18	13.4	17.5	1.42 (0.72, 2.79)		1.19 (0.53, 2.68)	
	Category 4: ≥6.19	25.0	27.8	1.21 (0.68, 2.16)		1.16 (0.53, 2.68)	
Meat Mutagenicity	Category 1: ≤3589.83	25.5	24.6	Referent	0.43	Referent	0.81
	Category 2: 3589.84-7179.67	33.3	26.2	0.81 (0.45, 1.49)		0.85 (0.42, 1.76)	
	Category 3: 7179.68-10769.50	16.2	21.4	1.37 (0.70, 2.67)		1.03 (0.46, 2.34)	
	Category 4: ≥10769.51	25.0	27.8	1.15 (0.62, 2.12)		1.20 (0.57, 2.53)	

[1]p-value obtained from a Wald Chi-Square Statistic

[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio

Table 7.13. Multivariable analysis of colorectal adenoma risk associated with dietary HAA intake and exposure to meat mutagenicity as represented by a dichotomous variable

	Categories (ng/day)	% Non-events (N=213)	% Events (N=123)	Crude OR (95% CI)	p[1]	Adjusted OR[2] (95% CI)	p[1]
PhIP	Category 1, 2 & 3: <348.92	75.0	70.6	Referent	0.38	Referent	0.45
	Category 4: ≥348.92	25.0	29.4	1.25 (0.76, 2.04)		1.26 (0.69, 2.29)	
MeIQx	Category 1, 2 & 3: <55.64	75.0	73.8	Referent	0.81	Referent	0.99
	Category 4: ≥55.64	25.0	26.2	1.07 (0.64, 1.76)		1.00 (0.55, 1.81)	
DiMeIQx	Category 1, 2 & 3: <6.19	75.0	72.2	Referent	0.41	Referent	0.49
	Category 4: ≥6.19	25.0	27.8	1.27 (0.72, 2.26)		1.23 (0.69, 2.20)	
Meat Mutagenicity	Category 1, 2 & 3: <10769.51	75.0	27.8	Referent	0.57	Referent	0.42
	Category 4: ≥10769.51	25.0	72.2	1.15 (0.70, 1.90)		1.27 (0.71, 2.28)	

[1]p-value obtained from a Wald Chi-Square Statistic
[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio

7.7 Genetic Analysis

7.7.1 Genetic Determinants of Colorectal Adenoma

The multivariable relationships between genetic factors and colorectal adenoma risk were examined using multiple logistic regression. The main effects of each genetic factor were individually assessed in separate models while controlling for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker levels of albumin, folate and vitamin B_{12}, and lipid ratio (Table 7.14). In multivariable analyses, the haplotype representation of CYP1B1 (rs10012 and rs1056827) was associated with colorectal adenoma risk (OR=2.29, 95% CI: 1.30-4.04). As well, XPC rs228001 was associated with colorectal adenoma risk, in that those with XPC "CC or CA" genotypes were found to have an OR of 1.76 (p=0.05) for colorectal adenoma.

Table 7.14. Multivariable analysis of colorectal adenoma risk associated with genetic factors

Gene	rs	Genotype Category	Adjusted OR[1] (95% CI)	p[2]
CYP1A2	762551	AA AC/CC	0.87 (0.51, 1.51) Referent	0.63
CYP1B1	10012/1056827	CG/GG+GT/TT CC+GG	2.50 (1.38, 4.53) Referent	<0.01
NAT2	1041983	CC/CT TT	1.24 (0.73, 2.11) Referent	0.43
XPA	1800975	CC CT/TT	0.65 (0.38, 1.10) Referent	0.10
XPD	13181	CC/CA AA	1.70 (0.97, 2.99) Referent	0.06
XPD	1799793	AA/GA GG	0.89 (0.52, 1.52) Referent	0.67
XRCC1	25487	AA/AG GG	0.91 (0.53, 1.57) Referent	0.74
ADPRT	1136410	CC/CT TT	1.04 (0.59, 1.86) Referent	0.88
XPC	2228001	CC/CA AA	1.76 (1.00, 3.08) Referent	0.05
XRCC3	861539	TT/TC CC	0.74 (0.43, 1.30) Referent	0.30

[1]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[2]p-value obtained from a Wald Chi-Square Statistic

7.7.2 Gene-Diet Interaction Analysis

For gene-diet interactions, all HAAs and meat mutagenicity exposures were represented using dichotomous representations based on combining categories 3 and 4 and utilizing categories 1 and 2 as the referent; the effects of HAAs and meat mutagenicity on colorectal adenoma risk within strata of genetic factors were assessed. Tables 7.15 to 7.18 present the gene-diet results assessed in adjusted logistic regression models controlling for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio.

For PhIP, interactions with CYP1B1 (rs10012 and rs1056827), XPD (rs13181) and XPD (rs1799793) on colorectal adenoma risk were observed. Specifically, among those with the CYP1B1 rs10012 'CG or GG' and rs1056827 'GT or TT' genotypes which confer higher bioactivation activity, higher PhIP intake was associated with an increased risk of colorectal adenoma (OR=2.43; 95% CI: 1.00-5.88). For XPD (rs13181) and XPD (rs1799793), among genotypes which confer lower DNA repair activity (XPD rs13181 'CC or CA' and/or XPD rs1799793 'AA or AG' genotypes), higher PhIP intake was associated with a higher risk of colorectal adenoma (XPD rs13181: OR=1.85; 95% CI: 0.91-3.76 and XPD rs1799793: OR=1.84; 95% CI 0.87-3.88). Similar gene-diet interactions were observed for DiMeIQx and polymorphisms in XPD (rs13181 and rs1799793), and meat mutagenicity and CYP1B1 (rs10012 and rs1056827) and XPD (rs13181) polymorphisms. No significant interactions were observed for MeIQx.

Table 7.15. Multivariable analysis of colorectal adenoma risk associated with dietary PhIP intake stratified by genetic factors

Gene	rs	Genotype Category	Categories 3 and 4 PhIP>232.61 ng/day[1] Adjusted OR[2] (95% CI)	p[3]
CYP1A2	762551	AA	1.53 (0.69, 3.38)	0.13
		AC/CC	0.65 (0.30, 1.42)	
CYP1B1	10012/1056827	CG/GG+GT/TT	2.43 (1.00, 5.88)	<0.01
		CC+GG	0.44 (0.19, 1.03)	
NAT2	1041983	CC/CT	0.88 (0.41, 1.91)	0.71
		TT	1.08 (0.50, 2.33)	
XPA	1800975	CC	1.04 (0.47, 2.31)	0.79
		CT/TT	0.90 (0.44, 1.85)	
XPD	13181	CC/CA	1.85 (0.91, 3.76)	<0.01
		AA	0.33 (0.13, 0.85)	
XPD	1799793	AA/GA	1.84 (0.87, 3.88)	0.02
		GG	0.46 (0.20, 1.06)	
XRCC1	25487	AA/AG	1.23 (0.58, 2.64)	0.35
		GG	0.72 (0.31, 1.66)	
ADPRT	1136410	CC/CT	1.07 (0.42, 2.75)	0.83
		TT	0.94 (0.48, 1.86)	
XPC	2228001	CC/CA	1.13 (0.56, 2.26)	0.68
		AA	0.89 (0.35, 2.24)	
XRCC3	861539	TT/TC	0.84 (0.41, 1.72)	0.31
		CC	1.51 (0.61, 3.73)	

[1]Referent category: Categories 1 and 2 of PhIP intake ≤232.61 ng/day
[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[3]p-value obtained from a Wald Chi-Square Statistic

Table 7.16. Multivariable analysis of colorectal adenoma risk associated with dietary MeIQx intake stratified by genetic factors

Gene	rs	Genotype Category	Categories 3 and 4 MeIQx>31.09 ng/day[1] Adjusted OR[2] (95% CI)	p[3]
CYP1A2	762551	AA	2.25 (1.02, 4.99)	0.13
		AC/CC	0.97 (0.45, 2.07)	
CYP1B1	10012/1056827	CG/GG+GT/TT	1.93 (0.85, 4.39)	0.17
		CC+GG	0.86 (0.38, 1.98)	
NAT2	1041983	CC/CT	1.41 (0.66, 3.03)	0.94
		TT	1.47 (0.68, 3.18)	
XPA	1800975	CC	1.56 (0.71, 3.44)	0.61
		CT/TT	1.19 (0.58, 2.42)	
XPD	13181	CC/CA	2.09 (1.04, 4.21)	0.08
		AA	0.75 (0.30, 1.84)	
XPD	1799793	AA/GA	2.29 (1.09, 4.81)	0.08
		GG	0.85 (0.38, 1.91)	
XRCC1	25487	AA/AG	1.45 (0.68, 3.07)	0.99
		GG	1.46 (0.65, 3.27)	
ADPRT	1136410	CC/CT	1.06 (0.43, 2.65)	0.41
		TT	1.71 (0.87, 3.37)	
XPC	2228001	CC/CA	1.79 (0.91, 3.52)	0.34
		AA	1.02 (0.40, 2.62)	
XRCC3	861539	TT/TC	1.37 (0.68, 2.78)	0.61
		CC	(0.75, 4.56)	

[1]Referent category: Categories 1 and 2 of MeIQx intake≤37.09 ng/day
[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[3]p-value obtained from a Wald Chi-Square Statistic

Table 7.17 Multivariable analysis of adenoma risk associated with dietary DiMeIQx intake stratified by genetic factors

Gene	rs	Genotype Category	Categories 3 and 4 DiMeIQx>4.12 ng/day[1] Adjusted OR[2] (95% CI)	p[3]
CYP1A2	762551	AA	1.75 (0.80, 3.85)	0.46
		AC/CC	1.15 (0.54, 2.47)	
CYP1B1	10012/1056827	CG/GG+GT/TT	1.95 (0.85, 4.47)	0.16
		CC+GG	0.84 (0.37, 1.93)	
NAT2	1041983	CC/CT	1.60 (0.74, 3.48)	0.65
		TT	1.24 (0.58, 2.66)	
XPA	1800975	CC	2.09 (0.96, 4.58)	0.11
		CT/TT	0.88 (0.43, 1.81)	
XPD	13181	CC/CA	2.13 (1.06, 4.26)	0.04
		AA	0.62 (0.25, 1.57)	
XPD	1799793	AA/GA	2.36 (1.13, 4.93)	0.04
		GG	0.75 (0.33, 1.70)	
XRCC1	25487	AA/AG	1.13 (0.54, 2.35)	0.45
		GG	1.73 (0.76, 3.95)	
ADPRT	1136410	CC/CT	0.82 (0.32, 2.09)	0.16
		TT	1.88 (0.95, 3.71)	
XPC	2228001	CC/CA	1.70 (0.87, 3.35)	0.43
		AA	1.07 (0.42, 2.74)	
XRCC3	861539	TT/TC	1.50 (0.74, 3.02)	0.65
		CC	1.16 (0.47, 2.82)	

[1]Referent category: Categories 1 and 2 of DiMeIQx intake≤4.12 ng/day
[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[3]p-value obtained from a Wald Chi-Square Statistic

Table 7.18. Multivariable analysis of adenoma risk associated with meat mutagenicity exposure stratified by genetic factors

Gene	rs	Genotype Category	Categories 3 and 4 Meat Mutagenicity >7179.67 ng/day[1] Adjusted OR[2] (95% CI)	p[3]
CYP1A2	762551	AA	1.68 (0.77, 3.69)	0.31
		AC/CC	0.95 (0.44, 2.08)	
CYP1B1	10012/1056827	CG/GG+GT/TT	3.65 (1.48, 9.02)	<0.01
		CC+GG	0.50 (0.21, 1.17)	
NAT2	1041983	CC/CT	1.29 (0.60, 2.79)	0.92
		TT	1.22 (0.57, 2.62)	
XPA	1800975	CC	1.15 (0.51, 2.58)	0.92
		CT/TT	1.22 (0.60, 2.47)	
XPD	13181	CC/CA	2.25 (1.10, 4.61)	<0.01
		AA	0.43 (0.17, 1.10)	
XPD	1799793	AA/GA	1.92 (0.91, 4.06)	0.10
		GG	0.77 (0.34, 1.74)	
XRCC1	25487	AA/AG	1.14 (0.54, 2.39)	0.76
		GG	1.43 (0.63, 3.27)	
ADPRT	1136410	CC/CT	1.43 (0.56, 3.65)	0.74
		TT	1.17 (0.60, 2.31)	
XPC	2228001	CC/CA	1.44 (0.73, 2.84)	0.65
		AA	1.10 (0.43, 2.81)	
XRCC3	861539	TT/TC	0.97 (0.47, 1.99)	0.14
		CC	2.29 (0.93, 5.67)	

[1]Referent category: Categories 1 and 2 of meat mutagenicity exposure ≤7179.67 ng/day
[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[3]p-value obtained from a Wald Chi-Square Statistic

7.8 Regression Diagnostics

Residual analysis was undertaken to assess the appropriateness of the logistic regression model predicting colorectal adenoma risk. Specifically, observations that were identified as outliers or those with a high leverage or influence were investigated for data entry errors and

plausibility was considered. The influence of these observations was also investigated by removal of each observation from the analysis; no difference in study results was observed.

7.9 Additional Analysis

7.9.1 Sex-specific Analysis

An analysis was conducted to evaluate sex-specific associations between dietary HAAs/meat mutagenicity and colorectal adenoma risk. Sex-specific analyses are presented in Tables 7.19 to 7.21. In Table 7.19, the associations between continuous representations of PhIP and meat mutagenicity exposures on colorectal adenoma risk were found to differ by sex; specifically, positive associations were observed only among males. In Table 7.20, utilizing equal-distant representations, the effect modification by sex were attenuated for the PhIP- or meat mutagenicity-colorectal adenoma relationships (p=0.06); however the positive trends between increasing exposure to PhIP or meat mutagenicity and colorectal adenoma risk was still observed only in males. In dichotomous representations, Table 7.21 presents statistically significant interactions with sex for DiMeIQx and meat mutagenicity; high intake of DiMeIQx (p=0.01) or high meat mutagencitiy (p<0.01) was associated with an increased risk of colorectal adenoma among males but not in females.

Table 7.19. Multivariable analysis of colorectal adenoma risk associated with dietary HAA intake (per SD) and exposure to meat mutagenicity by sex

Exposure	Females Adjusted OR[1] (95% CI)	Males Adjusted OR[1] (95% CI)	p[2]
PhIP (per SD)	0.91 (0.67, 1.25)	1.88 (1.01, 3.49)	0.04
MeIQx (per SD)	1.01 (0.76, 1.33)	1.52 (0.98, 2.36)	0.12
DiMeIQx (per SD)	1.01 (0.78, 1.32)	1.42 (0.94, 2.13)	0.17
Meat Mutagenicity (per SD)	0.90 (0.67, 1.22)	2.04 (1.16, 3.59)	0.01

[1]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[2]p-value for interaction term

Table 7.20. Multivariable analysis of colorectal adenoma risk associated with dietary HAA intake and exposure to meat mutagenicity as represented by a 4-level equal-distant categories by sex

	Categories (ng/day)	**Females Adjusted OR[1] (95% CI)**	**Males Adjusted OR[1] (95% CI)**	**p[2]**
PhIP	Category 1: ≤116.30	Referent	Referent	0.06
	Category 2: 116.31-232.61	0.41 (0.15, 1.14)	1.72 (0.63, 4.66)	
	Category 3: 232.62-348.91	0.29 (0.09, 0.98)	1.72 (0.52, 5.66)	
	Category 4: ≥348.92	0.48 (0.19, 1.24)	2.80 (0.95, 8.31)	
MeIQx	Category 1: ≤18.54	Referent	Referent	0.20
	Category 2: 18.55-37.09	1.96 (0.73, 5.25)	1.00 (0.36, 2.76)	
	Category 3: 37.10-55.63	1.99 (0.64, 6.18)	2.43 (0.79, 7.46)	
	Category 4: ≥55.64	0.85 (0.30, 2.41)	2.22 (0.79, 6.28)	
DiMeIQx	Category 1: ≤2.06	Referent	Referent	0.09
	Category 2: 2.07-4.12	0.76 (0.28, 2.06)	0.84 (0.30, 2.34)	
	Category 3: 4.13-6.18	1.55 (0.53, 4.54)	0.92 (0.29, 2.92)	
	Category 4: ≥6.19	0.54 (0.20, 1.51)	2.39 (0.85, 6.72)	
Meat Mutagenicity	Category 1: ≤3589.83	Referent	Referent	0.06
	Category 2: 3589.84-7179.67	0.92 (0.35, 2.38)	0.87 (0.31, 2.50)	
	Category 3: 7179.68-10769.50	1.03 (0.32, 3.35)	1.20 (0.39, 3.69)	
	Category 4: ≥10769.51	0.54 (0.19, 1.53)	3.23 (1.02, 10.28)	

[1]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[2]p-value for interaction term

Table 7.21. Multivariable analysis of colorectal adenoma risk associated with dietary HAA intake and exposure to meat mutagenicity as represented by a dichotomous variable by sex

	Categories (ng/day)	Females Adjusted OR[1] (95% CI)	Males Adjusted OR[1] (95% CI)	p[2]
PhIP	Category 1, 2 & 3: <348.92 Category 4: ≥348.92	Referent 0.85 (0.37-1.93)	Referent 2.03 (0.82-4.98)	0.16
MeIQx	Category 1, 2 & 3: <55.64 Category 4: ≥55.64	Referent 0.57 (0.24-1.37)	Referent 1.74 (0.74-4.12)	0.07
DiMeIQx	Category 1, 2 & 3: <6.19 Category 4: ≥6.19	Referent 0.54 (0.22-1.34)	Referent 2.65 (1.13-6.21)	0.01
Meat Mutagenicity	Category 1, 2 & 3: <10769.51 Category 4: ≥10769.51	Referent 0.56 (0.23-1.33)	Referent 3.24 (1.29-8.10)	<0.01

[1]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[2]p-value for interaction term

7.9.2 Use of OR for Prevalent Outcomes

To evaluate the extent of overestimation in the use of OR with a prevalent outcome, an additional analysis was conducted to compare the OR and prevalence ratio (PR) derived from a logistic and a weighted modified Poisson regression model with a robust error variance estimator based on the full study population (N=370). Table 7.22 presents the main effects of HAAs or meat mutagenicitiy on colorectal adenoma risk modeled using logistic regression and a weighted Poission regression with a robust variance estimator; the OR overestimated the PR in all models. Table 7.23 presents the OR and PR for the gene-diet interactions for dietary PhIP on colorectal adenoma risk. Like the main effects, the OR was found to overestimate the PR, for example, the adjusted PR for the risk of colorectal adenoma associated with higher intakes of dietary PhIP was found to be 1.51 times greater among those in the CYP1B1 ‘CG/GG+GT/TT’ category in contrast to an OR of 2.43 for the same contrast.

Table 7.22. Contrasting the main effects of HAAs or meat mutagenicity on colorectal adenoma risk using OR modelled using logistic regression versus PR modelled using a weighted Poisson regression with a robust variance estimator

	Categories (ng/day)	**Original Logistic Regression Adjusted OR[1] (95% CI)**	**p[2]**	**Weighted Poisson Regression Adjusted PR[1] (95% CI)**	**p[2]**
PhIP	Category 1: ≤116.30	Referent	0.65	Referent	0.58
	Category 2: 116.31-232.61	0.77 (0.38, 1.55)		0.86 (0.59, 1.26)	
	Category 3: 232.62-348.91	0.66 (0.29, 1.51)		0.81 (0.52, 1.29)	
	Category 4: ≥348.92	1.03 (0.50, 2.11)		1.04 (0.71, 1.54)	
MeIQx	Category 1: ≤18.54	Referent	0.29	Referent	0.20
	Category 2: 18.55-37.09	1.37 (0.67, 2.83)		1.16 (0.78, 1.73)	
	Category 3: 37.10-55.63	2.19 (0.99, 4.81)		1.54 (1.05, 2.25)	
	Category 4: ≥55.64	1.37 (0.99, 4.81)		1.22 (0.83, 1.82)	
DiMeIQx	Category 1: ≤2.06	Referent	0.68	Referent	0.63
	Category 2: 2.07-4.12	0.78 (0.38, 1.61)		0.90 (0.61, 1.33)	
	Category 3: 4.13-6.18	1.19 (0.53, 2.68)		1.11 (0.75, 1.64)	
	Category 4: ≥6.19	1.16 (0.53, 2.68)		1.14 (0.79, 1.65)	
Mutagen	Category 1: ≤3589.83	Referent	0.81	Referent	0.76
	Category 2: 3589.84-7179.67	0.85 (0.42, 1.76)		0.92 (0.61, 1.40)	
	Category 3: 7179.68-10769.50	1.03 (0.46, 2.34)		1.03 (0.67, 1.56)	
	Category 4: ≥10769.51	1.20 (0.57, 2.53)		1.14 (0.77, 1.67)	

[1]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio

[2]p-value obtained from a Wald Chi-Square Statistic

Table 7.23. Sensitivity analysis contrasting the gene-diet interactions of PhIP and polymorphisms in CYP1B1 and XPD on colorectal adenoma risk using OR modelled using logistic regression versus PR modelled using a weighted Poisson regression with a robust variance estimator

Gene	**rs**	**Genotype Category**	**Categories 3 and 4 PhIP>232.61 ng/day [1] Original Logistic Regression Adjusted OR[2] (95% CI)**	**p[3]**	**Categories 3 and 4 PhIP>232.61 ng/day[1] Weighted Poisson Regression Adjusted PR[2] (95% CI)**	**p[3]**
CYP1B1	10012/	CG/GG+GT/TT	2.43 (1.00, 5.88)	<0.01	1.51 (1.00, 2.27)	<0.01
	1056827	CC+GG	0.44 (0.19, 1.03)		0.68 (0.44, 1.06)	
XPD	13181	CC/CA	1.85 (0.91, 3.76)	<0.01	1.35 (0.95, 1.93)	<0.01
		AA	0.33 (0.13, 0.85)		0.58 (0.35, 0.96)	
XPD	1799793	AA/GA	1.84 (0.87, 3.88)	0.02	1.36 (0.91, 2.03)	0.02
		GG	0.46 (0.20, 1.06)		0.72 (0.47, 1.09)	

[1]Exposure categorized based on 4-level equal-distant categories among non-event group. Referent category: Categories 1 and 2 of PhIP intake≤232.61 ng/day

[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lip

[3]p-value obtained from a Wald Chi-Square Statistic

7.10 Sensitivity Analysis

7.10.1 Alternative Confounder Selection Approach

Stepwise selection was used to create a parsimonious model of covariates that were predictive of colorectal adenomas using a liberal p-value of 0.20. Predictors of colorectal adenoma were included in all multivariable analyses. To evaluate the confounder selection method employed, the change-in-estimate method was used to identify confounders among the parsimonious model of covariates ($p \leq 0.20$) for equal-distant representation of PhIP using the OR of Category 4 versus Category 1 of PhIP intake as the parameter estimate of interest in the main effect analysis. In the main effects model, the confounders identified from the change-in-estimate procedure included biomarker levels of albumin and lipid ratio. The ORs for colorectal adenoma associated with the main effects of PhIP and corresponding 95% CI derived from each approach are presented in Table 7.24. Results for the main effects of PhIP on colorectal adenoma risk are similar between both approaches. In Table 7.25, the gene-PhIP interactions on colorectal adenoma risk are contrasted using the parsimonious versus change-in parameter estimate approach. The change-in-estimate procedure utilized the OR in categories 3 and 4 versus categories 1 and 2 of PhIP intake in those with higher bioactivation (CYP1B1 rs10012 'CG or GG' and rs1056827 'GT or TT' genotypes) and lower DNA repair activity (XPD rs13181 CC or CA genotypes and/or XPD rs1799793 'AA or AG' genotypes) as the parameter estimates of interest. Results for the gene-diet interactions on colorectal adenoma risk are similar between both approaches.

Table 7.24. Sensitivity analysis – main effects of PhIP using different confounder selection. strategies

PhIP (ng/day)	Parsimonious Approach Adjusted OR[1] (95% CI)	Change-in-Estimate Approach Adjusted OR[2] (95% CI)
Category 1: ≤116.30	Referent	Referent
Category 2: 116.31-232.61	0.77 (0.38, 1.55)	0.90 (0.48, 1.66)
Category 3: 232.62-348.91	0.66 (0.29, 1.51)	0.82 (0.39, 1.70)
Category 4: >348.92	1.03 (0.50, 2.11)	1.07 (0.58, 2.00)
p[3]	0.65	0.52

[1]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[2]Adjusted for biomarker levels of albumin and lipid ratio.
[3]p-value obtained from a Wald Chi-Square Statistic

Table 7.25. Sensitivity analysis: gene-PhIP interactions using different confounder selection strategies

Gene	rs	Genotype Category	Categories 3 and 4 PhIP>232.61 ng/day[1] Parsimonious Approach Adjusted OR[2] (95% CI)	p[3]	Categories 3 and 4 PhIP>232.61 ng/day[1] Change-in-Estimate Approach Adjusted OR[4] (95% CI)	p[3]
CYP1B1	10012/ 1056827	CG/GG+GT/TT	2.43 (1.00, 5.88)	<0.01	2.41 (1.03, 5.65)	0.01
		CC+GG	0.44 (0.19, 1.03)		0.53 (0.24, 1.19)	
XPD	13181	CC/CA	1.85 (0.91, 3.76)	<0.01	2.00 (1.05, 3.81)	<0.01
		AA	0.33 (0.13, 0.85)		0.45 (0.19, 1.08)	
XPD	1799793	AA/GA	1.84 (0.87, 3.88)	0.02	1.68 (0.85, 3.32)	0.04
		GG	0.46 (0.20, 1.06)		0.57 (0.26, 1.23)	

[1]Referent category: Categories 1 and 2 of PhIP intake≤232.61 ng/day
[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
[3]p-value obtained from a Wald Chi-Square Statistic
[4]CYP1B1 analysis adjusted for sex, previous diagnosis of IBS, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio
XPD (rs13181) analysis adjusted for sex, previous diagnosis of IBS and biomarker level of albumin, vitamin B_{12} and lipid ratio
XPD (rs1799793) analysis adjusted for sex, fruit and vegetable intake and biomarker level of albumin and lipid ratio

7.10.2 Impact of Adjustment for Total Energy Intake

A conventional regression method (i.e. stepwise selection) was used to build a parsimonious model predicting colorectal adenoma risk. Using this procedure, sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio were determined to be predictors of colorectal adenoma risk at a 0.20 level of statistical significance. A sensitivity analysis was conducted to assess the impact of adjusting for total energy intake; total energy intake fits the definition of a confounder since the level of energy intake may be a primary determinant of colorectal adenoma risk, is likely to be correlated with dietary HAA exposures but is not on the causal pathway between HAA and colorectal adenoma. Table 7.26 presents the main effects of specific HAAs and meat mutagenicity (equal-distant representations) on colorectal adenoma risk with and without adjustment for total energy intake; no differences in main effects were observed. Tables 7.27 presents the gene-diet interactions for PhIP on colorectal adenoma risk with and without adjustment for total energy intake; no differences between the two models were observed. The impact of adjustment for total energy intake on gene-diet interactions for MeIQx, DiMeIQx and meat mutagenicity did not yield any results that differed from unadjusted models and thus, results are not presented.

Table 7.26. Sensitivity analysis – main effects of HAAs and meat mutagenicity on colorectal adenoma risk with and without adjustment for total energy intake.

	Categories (ng/day)	No Adjustment for Energy Intake[1] Adjusted OR[1] (95% CI)	p[2]	Adjustment for Energy Intake[3] Adjusted OR[3] (95% CI)	p[2]
PhIP	Category 1: ≤116.30	Referent	0.65	Referent	0.67
	Category 2: 116.31-232.61	0.77 (0.38, 1.55)		0.77 (0.38, 1.55)	
	Category 3: 232.62-348.91	0.66 (0.29, 1.51)		0.67 (0.29, 1.56)	
	Category 4: ≥348.92	1.03 (0.50, 2.11)		1.03 (0.50, 2.11)	
MeIQx	Category 1: ≤18.54	Referent	0.29	Referent	0.28
	Category 2: 18.55-37.09	1.37 (0.67, 2.83)		1.36 (0.66, 2.79)	
	Category 3: 37.10-55.63	2.19 (0.99, 4.81)		2.21 (1.00, 4.88)	
	Category 4: ≥55.64	1.37 (0.67, 2.83)		1.32 (0.64, 2.72)	
DiMeIQx	Category 1: ≤2.06	Referent	0.68	Referent	0.70
	Category 2: 2.07-4.12	0.78 (0.38, 1.61)		0.78 (0.38, 1.62)	
	Category 3: 4.13-6.18	1.19 (0.53, 2.68)		1.18 (0.52, 2.66)	
	Category 4: ≥6.19	1.16 (0.58, 2.35)		1.16 (0.58, 2.35)	
Meat Mutagenicity	Category 1: ≤3589.83	Referent	0.81	Referent	0.82
	Category 2: 3589.84-7179.67	0.85 (0.42, 1.76)		0.86 (0.42, 1.78)	
	Category 3: 7179.68-10769.50	1.03 (0.46, 2.34)		1.05 (0.46, 2.38)	
	Category 4: ≥10769.51	1.20 (0.57, 2.53)		1.21 (0.57, 2.54)	

[1]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio

[2]p-value obtained from a Wald Chi-Square Statistic

[3]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and biomarker level of albumin, folate, vitamin B_{12} and lipid ratio and total energy intake

Table 7.27. Sensitivity analysis: gene-PhIP interaction on colorectal adenoma risk with and without adjustment for total energy intake

Gene	rs	Genotype Category	Categories 3 and 4 PhIP>232.61 ng/day[1] Without Adjustment[2] Adjusted OR[2] (95% CI)	p[3]	Categories 3 and 4 PhIP>232.61 ng/day [1] With Adjustment[2] Adjusted OR[2] (95% CI)	p[3]
CYP1A2	762551	AA	1.53 (0.69, 3.38)	0.13	1.56 (0.70, 3.47)	0.12
		AC/CC	0.65 (0.30, 1.42)		0.65 (0.30, 1.41)	
CYP1B1	10012/ 1056827	CG/GG+GT/TT	2.43 (1.00, 5.88)	<0.01	2.45 (1.01, 5.97)	<0.01
		CC+GG	0.44 (0.19, 1.03)		0.45 (0.19, 1.06)	
NAT2	1041983	CC/CT	0.88 (0.41, 1.91)	0.71	0.90 (0.41, 1.94)	0.73
		TT	1.08 (0.50, 2.33)		1.08 (0.50, 2.33)	
XPA	1800975	CC	1.04 (0.47, 2.31)	0.79	1.09 (0.49, 2.42)	0.72
		CT/TT	0.90 (0.44, 1.85)		0.90 (0.43, 1.85)	
XPD	13181	CC/CA	1.85 (0.91, 3.76)	<0.01	1.92 (0.93, 3.93)	<0.01
		AA	0.33 (0.13, 0.85)		0.32 (0.13, 0.83)	
XPD	1799793	AA/GA	1.84 (0.87, 3.88)	0.02	1.89 (0.89, 4.00)	0.01
		GG	0.46 (0.20, 1.06)		0.45 (0.19, 1.05)	
XRCC1	25487	AA/AG	1.23 (0.58, 2.64)	0.35	1.24 (0.58, 2.66)	0.36
		GG	0.72 (0.31, 1.66)		0.73 (0.32, 1.70)	
ADPRT	1136410	CC/CT	1.07 (0.42, 2.75)	0.83	1.07 (0.41, 2.78)	0.98
		TT	0.94 (0.48, 1.86)		0.95 (0.48, 1.89)	
XPC	2228001	CC/CA	1.13 (0.56, 2.26)	0.68	1.14 (0.57, 2.29)	0.66
		AA	0.89 (0.35, 2.24)		0.88 (0.35, 2.25)	
XRCC3	861539	TT/TC	0.84 (0.41, 1.72)	0.31	0.85 (0.41, 1.75)	0.33
		CC	1.51 (0.61, 3.73)		1.50 (0.61, 3.73)	

[1]Exposure categorized based on 4-level equal-distant categories among non-event group. Referent: Categories 1 and 2 of PhIP intake≤232.61 ng/day

[2]Adjusted for sex, smoking status, previous diagnosis of IBS, fruit and vegetable intake, dietary protein intake and serum albumin, folate, vitamin B_{12} and lipid ratio

[3]p-value obtained from a Wald Chi-Square Statistic

7.11 Summary of Phase 2 Results

The frequent consumption of meats has been linked to an increased risk of CRC; one potential biologic explanation for this relationship is exposure to HAAs, a class of carcinogens formed when meat is cooked at high temperatures, which contributes to the total mutagenic activity of meat. However, there remain substantial inconsistencies in the epidemiologic literature regarding the HAA-CRC/colorectal adenoma association possibly due to the considerable inter-individual differences in susceptibility to HAAs. Variants in genes encoding enzymes involved in the biotransformation of HAAs and DNA repair may all play roles in determining an individual's risk of CRC. In this cross-sectional study of 342 individuals undergoing a screening colonoscopy, Phase 2 investigated the relationship between dietary exposure to HAAs and the prevalence of colorectal adenoma, precursors to the vast majority of CRCs. In addition, Phase 2 examined the effect of meat mutagenicity in relation to colorectal adenoma prevalence; meat mutagenicity provided a summary measure of exposure to <u>all</u> dietary HAAs as well as other meat-derived carcinogens. Effect modification by variation in genetic susceptibility to biotransform HAAs and repair DNA on the main relationships of interest was also examined.

No main effects were observed on the risk of developing colorectal adenomas for dietary HAAs. Polymorphisms in genes involved in the biotransformation of HAAs (CYP1B1 rs10012 and rs1056827) and DNA repair (XPC rs2228001) were found to contribute colorectal adenoma risk. As well, gene-diet interactions were observed for dietary PhIP and polymorphisms in CYP1B1 and XPD (rs13181 and rs1799793), dietary DiMeIQx and polymorphisms in XPD (rs13181, rs1799793), and meat mutagenicity and polymorphisms in CYP1B1 and XPD (rs13181). Overall, a higher risk of colorectal adenoma was observed with higher HAA and/or

meat mutagenicity exposure among those with polymorphisms expected to result in elevated HAA bioactivation and/or a lower ability to repair DNA damage. In an additional analysis conducted to evaluate the dietary HAAs-colorectal adenoma and meat mutagenicitiy-colorectal adenoma associations separately among males and females, strong positive relationships between dietary PhIP, DiMeIQx and meat mutagenicity and colorectal adenoma risk were observed in males only.

7.12 References

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Chapter 8: Discussion and Conclusion

8.1 Overview

This research investigated the contribution of dietary and genetic determinants to cancer risk using molecular epidemiologic approaches. Specifically, the roles of heterocyclic aromatic amines (HAAs), which are meat-derived carcinogens, and relevant genetic factors on colorectal adenoma risk were examined. The HAA-colorectal adenoma association was examined in two phases (Phase 1 and 2). In a cross-sectional study of 99 participants, Phase 1 investigated the relationship between dietary exposure to HAAs and the formation of potentially carcinogenic bulky DNA adducts in blood. In Phase 2, a cross-sectional study investigated the relationship between dietary exposure to HAAs and the prevalence of colorectal adenoma among 342 participants undergoing a screening colonoscopy. In addition, Phase 2 examined the effect of exposure to meat mutagenicity in relation to colorectal adenoma prevalence. Both Phase 1 and 2 examined gene-diet interactions between dietary HAAs and genetic factors in relation to bulky DNA adduct levels (outcome of interest for Phase 1) and colorectal adenoma risk (outcome of interest for Phase 2). This Chapter summarizes the findings of Phase 1 and 2, compares results to published literature, highlights the methodological strengths and limitations, and considers future research directions based on study findings.

8.2 Phase 1: Dietary HAA Intake and Bulky DNA Adducts

8.2.1 Summary of Key Findings

Phase 1 investigated the relationship between dietary exposure to HAAs and the prevalence of bulky DNA adducts in blood leukocytes in order to inform on a potential mechanism through which meat consumption may increase CRC risk. Interactions with genetic factors that have a role in the biotransformation of HAAs or DNA adduct repair were

investigated to potentially identify strong relationships in susceptible subgroups and contribute to biologic plausibility.

Though no statistically significant main effects were found for dietary HAA exposures on bulky DNA adduct levels; the highest category of intake for dietary PhIP, defined as greater than the 75th percentile, was suggestive of an increase in bulky DNA adducts when compared to those with lower intakes (p=0.10). In the genetic analyses, polymorphisms in NAT1 were found to relate to bulky DNA adduct levels such that, among those without the NAT1 variant alleles (the putative slow acetylator genotype), higher DNA adduct levels were observed. Hence, the genotype hypothesized to produce lower levels of HAA acetylation (based on functional evidence for enzymatic activity), was associated with higher levels of DNA adducts. A significant interaction was observed for dietary HAAs and NAT1 polymorphisms supporting the importance of both dietary and genetic influences on bulky DNA adduct formation. Furthermore, the highest level of DNA adducts was observed in those in the under/normal weight body mass index (BMI) category.

8.2.2 Comparison to the Literature

Phase 1 is a novel investigation of the relationship between dietary exposure to HAAs and the formation of potentially carcinogenic bulky DNA adduct levels in blood leukocytes. Although no published study has investigated the association between dietary exposure to HAAs and bulky DNA adduct levels in blood, four papers investigated other dietary determinants of bulky DNA adducts with a focus on broad patterns of meat consumption (1-4). Two of the four studies found a positive association between dietary meat intake and bulky DNA adduct levels (1-4).

Several studies have investigated genetic determinants of bulky DNA adducts in blood leukocytes among healthy individuals (5-10); however, this is the first study to examine this large subset of relevant genetic factors involved in the biotransformation of HAAs and the repair of DNA adducts. Only one previous study investigated polymorphisms in NAT1 as potential genetic determinants of bulky DNA adducts in blood leukocytes. The results of this study conducted among smokers by Godschalk et al. support the NAT1 finding observed in Phase 1; specifically, slow acetylators were found to have higher adduct levels than fast acetylators (11). No published study has evaluated gene-diet interactions between dietary HAAs and genetic factors on bulky DNA adduct formation in blood leukocytes; thus this is the first study to report an interaction between dietary HAAs and NAT1 polymorphisms in determining bulky DNA adduct levels.

Six studies have previously reported on the association between BMI and bulky DNA adducts (3,4,12-15); five of which support the trend of decreasing DNA adducts with increasing BMI observed in Phase 1 (3,4,12-15).

8.2.3 Consideration and Hypothesis of Underlying Biologic Relationships

The lack of an independent relationship between dietary exposure to HAAs and bulky DNA adduct levels could represent a true association, where the formation of DNA adducts is not a primary mechanism through which HAAs may contribute to carcinogenesis; or alternatively, the measure of bulky DNA adducts may not adequately capture specific HAA-DNA adducts in leukocytes. Furthermore, it is also possible that a one-time measure of bulky DNA adducts either does not reflect steady-state concentrations or does not capture the biologically relevant time window of exposure to dietary HAAs within the previous year. The relationship between dietary HAA intake and bulky DNA adduct levels was demonstrated to be

modified by genetic susceptibility as conferred by NAT1 polymorphisms. However, other dietary constituents (16) or cooking habits (17) not considered in Phase 1 may also modify the dietary HAA-bulky DNA adduct relationship and obscure the observed association (further discussed in Information Bias in Section 8.2.4).

NAT1 plays a role in the bioactivation of HAAs and other carcinogens in diet, cigarette smoke and the environment (18); specifically, subsequent to *N*-oxidation by cytochrome P450s, NAT1 conjugates the metabolite, to produce the ultimate carcinogenic metabolite that may covalently bind to DNA and form an adduct (refer to Chapter 2 Figure 2.3). At first glance, the directionality of having higher adduct levels among those without the NAT1 variant alleles (i.e. having the putative slow acetylator phenotype), is contrary to the functional evidence of the published impact of NAT1 polymorphisms on activity (19). If the association is true, this result could indicate the shortcoming of the NAT1 functional evidence in determining the genotype-phenotype correlations as they relate to the catalytic activity of NAT1 (19). On the other hand, genotyping alone usually is not a definitive representation of the *in vivo* situation; however it is unlikely that the influence of endogenous and exogenous factors on the regulation of the NAT1 gene and the activity of the enzyme could entirely account for the complete reversal of the predicted functional effect (17). As well, detoxification pathways operate alongside bioactivation processes; hence, it is possible that lower bioactivation activity (i.e. those without the NAT1 variant alleles or having the putative slow acetylator phenotype) may be linked to a lower ability to detoxify or excrete HAAs, thereby conferring higher DNA adduct levels.

This is the first study to examine and report significant interactions between dietary exposure to HAAs and NAT1 polymorphisms on determining bulky DNA adduct levels. Specifically, higher intakes of dietary HAAs were associated with higher levels of bulky DNA

adducts only among those with the NAT1 slow acetylator phenotype. The directionality of the dietary HAA-DNA adduct relationship within genetic subgroups defined by NAT1 provides some support for the role of bulky DNA adducts as a potential mechanism through which dietary exposure to HAAs may contribute to the carcinogenic process. Finally, the biological evidence to justify the observation of BMI as a determinant of bulky DNA adducts is scarce. It is unclear whether lower BMI is an independent risk factor for higher levels of bulky DNA adducts or whether dietary or genetic factors associated with lower BMI may predispose to the formation of adducts. Intuitively, it is possible that those with a lower BMI, given the same exposure to HAAs as those with a higher BMI, may have a higher body burden leading to a higher formation of bulky DNA adducts.

8.2.4 Methodological Considerations

Bias is defined as "the result of a systematic error in the design or conduct of a study" (20). Bias, either stemming from the selection of study participants (selection bias) or the procedures for obtaining and measuring variables of interest in a study (information bias), leads to systematic errors that cause the observed study results to deviate from the truth. The possibility of bias, in addition to that of confounding are important considerations in the interpretation of results in observational studies; the following sections review issues of temporality, selection bias, information bias and confounding specific to Phase 1.

i. Temporality

An often-cited limitation of cross-sectional studies is that the proper temporal sequence between a risk factor and a disease cannot be firmly established and thus precludes the establishment of causality (20). Temporal bias, a form of information bias (to be further discussed in Section iii), occurs when the inference regarding the temporal sequence of cause

and effect is erroneous. In Phase 1, the relationship between dietary intakes of HAAs in the past year and bulky DNA adduct levels in blood leukocytes was investigated. Though measures of exposure and outcome were cross-sectional, there is no evidence to support temporal bias since DNA adduct levels measured in blood at the study center could not have caused an individual to alter dietary HAA intakes over the previous year.

ii. Selection Bias

Selection bias, in the context of a cross-sectional study, occurs when systematic errors in the recruitment or participation of subjects differ between exposed and unexposed individuals resulting in a distortion of the exposure-outcome effect measure (20). As a consequence of selection bias, an effect measure is biased since the associations observed in the study would represent a mix of forces determining participation and disease.

In Phase 1, volunteers were recruited from Queen's University and its surrounding institutions to examine dietary HAAs in relation to bulky DNA adduct levels in blood leukocytes. Healthy behaviour is a potential factor that may influence study participation and relate to exposure (e.g. lower meat consumption) and outcome (e.g. lower bulky DNA adduct levels). However, since a biologic relationship was under investigation and participants were unaware of their bulky DNA adduct level, the use of healthy volunteers is not likely to have biased the observed associations.

iii. Information Bias

Information bias is a consequence of either imperfect definitions of study variables or flawed data collection procedures. Misclassification stemming from information bias may either be differential or non-differential. For Phase 1 of this research, dietary HAA exposures were ascertained independent of knowledge of the outcome of interest; thus, errors are independent of

outcome status and would result in non-differential misclassification in exposure assessment. Likewise, bulky DNA adducts were determined independent of exposure status, thus the inaccuracy or imprecision in measurement of DNA adducts is anticipated to cause misclassification to the same degree among comparison groups defined by dietary HAA exposures. The possibility of misclassification of confounding variables will be discussed in Section iv.

In Phase 1, the validity of the meat consumption module used to assess exposure to dietary HAAs was not assessed in our study population. However, the module used in Phase 1 was adapted from a questionnaire that was previously validated in a population of controls participating in a breast and prostate cancer case-control study in Bethesda, Maryland. Dietary habits were not anticipated to be hugely dissimilar between the Phase 1 study population and this population of controls; thus, results of this validation study are considered relevant and applicable to the interpretation of the results from Phase 1 (21).

Briefly, in the meat consumption module of Phase 1, each participant detailed the frequency of intake of specific types of meat cooked different ways, their usual doneness level and their usual serving size. Efforts were made to standardize the reporting of doneness level and serving size with the inclusion of photographs to the meat module. Using the Computerized Heterocyclic Amines Resource for Research in Epidemiology of Disease (CHARRED) mutagen database, the mean concentration of HAAs was attributed to the reported intakes and adjusted by serving size. The meat consumption module was adapted from existing food frequency questionnaires (FFQs) (see details in Chapter 4 Section 4.1.4) that had been previously validated by comparing the FFQ responses with three sets of four non-consecutive day food diaries distributed over a three-month period (21). Generally, the FFQ underestimated absolute HAA

intake, but given that its ability to rank an individual according to intake was acceptable, this FFQ has been used as an effective exposure assessment method in epidemiology studies (21). Specifically, individuals were classified by the FFQ and multiple diaries into the same or adjacent quintiles 70% of the time for MeIQx and 63% of the time for PhIP (DiMeIQx was not investigated in this study) (21). Misclassification into extreme quintiles (i.e. misclassification from Quintile 1 to Quintile 5 or vice versa) occurred in less than 6% (21).

It is well recognized that FFQs do not capture diet without some error based on the inaccuracy of recall of past exposures. In addition, the CHARRED mutagen database was developed based on a limited number of cooking assays and foods collected from a limited geographic area. Other factors including frequency of turning the meat over during the cooking process, meat thickness, cut of meat, use of marinade or thawing meat in the microwave were not captured in the questionnaire of Phase 1 and may have contributed to exposure variability (17). Overall, the inaccuracy of using an FFQ to estimate HAA exposure, limitations of the CHARRED mutagen database and additional sources of HAA variability not captured in our study may have led to non-differential misclassification. Non-differential misclassification would yield estimates that tended to be biased towards the null, although it is recognized that with two or more exposure categories biases in either direction are also a possibility (22).

The validity of the ^{32}P-postlabelling method in the measurement of bulky DNA adducts also deserves consideration. In the absence of a gold standard to measure the true quantity of bulky DNA adducts, reliability was assessed in place of validity. The coefficient of variation of the ^{32}P-postlabelling method was determined to be 17% indicating a potential for measurement error; however, random measurement error in a continuous outcome does not affect its regression on a predictor (23).

In genetic analyses, functional evidence was utilized to inform on the hypothesized effect of each polymorphism on enzymatic activity. However due to the limited sample size of Phase 1, heterozygotes were grouped with the homozygous variant category to maximize statistical efficiency. If the functional consequence of heterozygotes behaved more like the homozygous wild-type category then the *a priori* categorization may have introduced a degree of misclassification and limited the ability to detect an association if one existed.

iv. Confounding

Phase 1 collected information on established determinants of bulky DNA adducts; as well, efforts were made to systematically select the most appropriate variable representations of continuous covariates to minimize confounder misclassification and residual confounding. Confounder selection was conducted by building a parsimonious model predicting bulky DNA adduct levels in Phase 1. Relationships between dietary HAAs and covariates were examined to ensure that the parsimonious modeling strategy would capture the predictors of bulky DNA adducts that are also associated with dietary HAAs.

In Phase 1, the biologic relationship between exposure to dietary HAAs and bulky DNA adduct levels in blood leukocytes was examined. However, initially, the objective of Phase 1 was to identify and quantify specific HAA-DNA adducts. Thus, during the design of the study questionnaire, the assessment of factors that may influence HAA-DNA adduct formation was of interest; meat consumption patterns and cooking habits are the main determinants of HAA-DNA adduct formation. As a result, information was not collected for additional factors which may be important determinants of bulky DNA adducts.

Due to the failed validation attempts during the development of a mass spectrometric approach to identify and quantity specific HAA-DNA adducts (Appendix 1), bulky DNA adducts

were used as a surrogate measure in place of specific adducts. It was recognized that in addition to HAA-DNA adducts, a measure of bulky DNA adducts would encompass adducts formed from exposure to other genotoxicants like polycyclic aromatic hydrocarbons and nitrosamines. Thus, bulky DNA adduct levels may be influenced by additional lifestyle factors. Factors that have been reported to relate to bulky DNA adduct levels in blood include smoking, diet (fruit and vegetable consumption), BMI, seasonality, air pollution and polymorphisms in cytochrome P450 (CYP) 1A1, glutathione-S-transferase class M1 and T1, myeloperoxidase and NAD(P)H:quinone oxidoreductase (3,4,12-15). All factors with the exception of certain polymorphisms and air pollution were considered in Phase 1. However, due to the locality of the study area, air pollution was not expected to be an important contributor to bulky DNA adducts. As well, all participants were recruited within a limited geographic area which would have likely yielded little variation in exposure. Furthermore, air pollution is unlikely to be associated with dietary HAA exposure, mitigating the effects of unmeasured confounding due to air pollution

The consideration of variable representation for continuous covariates was prioritized due to the modest sample size of Phase 1. Three priorities were developed with the ultimate goal of minimizing residual confounding while balancing the parsimonious number of parameters for model building and interpretation of effects. These priorities led to a strategy of testing the fit of a continuous covariate using a quadratic term; evidence of a non-linear relationship was indicated by a partial F-test with a p-value of less than 0.10, and then categorical representation was selected in order to improve the clarity and interpretation of relationships. The use of categorical representations rather than polynomials may have contributed to a small degree of misclassification and introduced residual confounding.

In Phase 1, ethnicity, BMI, and fruit and vegetable intake were found to be determinants of bulky DNA adduct levels at a 0.20 level of significance; however only BMI was found to significantly predict DNA adduct levels in multivariable analysis ($p<0.05$). The main effects of dietary exposure to HAAs and bulky DNA adducts, while adjusting for the identified predictors did not differ appreciably from crude bivariate models.

Finally, to ensure that the parsimonious modeling strategy would capture the predictors of bulky DNA adducts that are also associated with dietary HAAs the examination of covariate associations with dietary HAAs, was undertaken. Bivariate analysis yielded an association between environmental tobacco smoke and dietary DiMeIQx intake. Strong correlations between PhIP, MeIQx and DiMeIQx were observed; but confounding was not assessed in order to avoid collinearity issues. However, the strong associations between different dietary HAAs, and the possibility to jointly predict bulky DNA adducts, could have led to residual confounding. Likewise, polycyclic aromatic hydrocarbons and N-nitroso compounds, other classes of meat carcinogens, were not considered in Phase 1; unmeasured confounding by other genotoxicants may have affected the results observed in Phase 1.

v. Statistical Power

In Phase 1, the modest sample size and the recruitment of healthy participants may have limited the ability to investigate the main effects of dietary HAAs on bulky DNA adduct levels in blood. Specifically, the distribution of bulky DNA adducts was fairly narrow, which may be due to the general healthy status of the study population. The size of the study sample limited the ability to examine gene-diet interactions; however despite statistical power limitations, interactions between dietary HAAs and NAT1 polymorphisms were observed.

8.3 Phase 2: Dietary HAA Intake and Risk of Colorectal Adenoma

8.3.1 Summary of Key Findings

In Phase 2, no main effects were observed on the risk of developing colorectal adenomas for individual exposures to dietary HAAs or meat mutagenicity; however, polymorphisms in genes involved in the bioactivation of HAAs (CYP1B1 rs10012 and rs1056827) and DNA repair (XPC rs2228001) were found to be associated with colorectal adenoma risk. Phase 2 found gene-diet interactions for dietary exposure to HAAs and polymorphisms in CYP1B1 (rs10012 and rs1056827) and XPD (rs13181 and XPD rs1799793). As well, effect modification by polymorphisms in CYP1B1 (rs10012 and rs1056827) and XPD (rs13181) on the meat mutagenicity-colorectal adenoma relationship was observed. Overall, a higher risk of colorectal adenoma was associated with higher HAA or meat mutagenicity exposures among those with polymorphisms that confer a greater genetic activity to bioactivate HAAs and/or a lower ability to repair DNA. In additional analyses, Phase 2 also investigated effect modification by sex on the dietary HAA/meat mutagenicity-colorectal adenoma association. A significant interaction was found where increasing exposure to dietary PhIP, dietary DiMeIQx and meat mutagenicity were associated with an increased risk of colorectal adenoma among males only.

8.3.2 Comparison to the Literature

Numerous studies have evaluated the relationship between dietary exposure to HAAs and CRC (17,24-37) or colorectal adenoma risk (16,17,24-35,38-40); 12 of which support a positive relationship, contrary to the findings of Phase 2. Likewise, nine studies have investigated the association between meat mutagenicity and colorectal adenoma risk; five of which report a positive association that was not observed in Phase 2. Phase 2 identified polymorphisms in *CYP1B1* and *XPC* as playing a role in determining colorectal adenoma risk. Four studies have

investigated polymorphisms in *CYP1B1* and CRC risk (41-44), two of which report an association (41,42). However, it is difficult to compare the results obtained in both of the previous studies to those observed in Phase 2 since one of the studies did not evaluate CYP1B1 rs1056827 (41) and the other had a different categorization of CYP1B1 genotypes (i.e. grouping rs10012 CC and rs1056827 TT genotypes) (42). For XPC rs2228001, a meta-analysis comprising 2,751 cases and 3,607 controls reported a higher risk of CRC associated with the XPC 'CC and CA' genotypes compared to 'AA,' which is consistent with our finding (45).

Few studies have investigated the modifying effects of this comprehensive subset of polymorphisms on the HAA/meat mutagenicity-colorectal adenoma associations. Five studies with varied overlap in the genetic factors of interest were identified (46-50) but none investigated polymorphisms in the CYP1B1 or XPD genes. This is the first study to report effect modification by polymorphisms in *CYP1B1* and *XPD* on the HAA/meat mutagenicity-colorectal adenoma relationships.

The sex-specific analysis was an additional examination of whether the HAA/meat mutagenicity-colorectal adenoma associations differed by sex. In Phase 2, sex-specific differences were observed, wherein positive associations between increasing exposures to PhIP, DiMeIQx and meat mutagenicity, and colorectal adenoma risk was observed only in males. This sex-specific difference has been previously investigated by five studies; one case-control study conducted in Hawaii among 727 matched pairs of cases and controls reported an interaction with sex (26).

8.3.3 Consideration and Hypothesis of Underlying Biologic Relationships

An independent role of dietary exposure to HAAs or meat mutagenicity in colorectal adenoma development is not supported by the findings of Phase 2. The lack of an association

may either indicate a true association, be a result of methodological limitations involved in exposure assessment (to be discussed in Section 8.4.2) or be suggestive of the role of effect modification by other dietary constituents (16) or individual characteristics that obscured the HAA/meat mutagenicity-colorectal adenoma relationships.

CYP1B1 and XPC play a role in the bioactivation of HAAs and the repair of damage induced by HAAs, respectively. Specifically, CYP1B1 functions in the bioactivation of HAAs in extrahepatic tissues leading to the formation of N-hydroxy-HAAs, which could undergo additional conjugation to form the ultimate carcinogenic metabolite. Throughout this process, if metabolites escape detoxification, metabolites of HAAs have been demonstrated to bind preferentially to the carbon-8 position of guanine in DNA, leading to the formation of DNA adducts. DNA adducts are repaired predominately by the nucleotide excision repair pathway; *XPC* encodes an enzyme that is involved in the damage recognition complex of nucleotide excision repair. Thus, though the functional effects of these proteins encoded by *CYP1B1* and *XPC* are not limited to HAAs, the relationship between polymorphisms in *CYP1B1* and *XPC* on colorectal adenoma risk would be consistent with the role of dietary HAAs in carcinogenesis.

The gene-diet interactions observed in Phase 2 between dietary HAAs/meat mutagenicity and polymorphisms in *CYP1B1* and *XPD* are all consistent with the functional evidence of each polymorphism, hence providing mechanistic support for the role of HAAs in CRC development through an influence on colorectal adenoma risk. *XPD* encodes a helicase enzyme that participates in the unwinding of the helix in the region of damaged DNA, further providing support for the role of DNA repair pathways in modifying the HAA-colorectal adenoma relationships.

The sex-specific relationship between dietary HAAs/meat mutagenicity and colorectal adenoma risk observed in Phase 2 was examined as an additional analysis due to an emerging hypothesis related to the inhibiting effects of estrogens on genetic factors like *CYP1A2* (51,52). Like the results observed in Phase 2, differences in sex-specific risks associated with cigarette smoking and CRC have also been reported in the literature; specifically, studies have shown that the heightened risk of CRC associated with cigarette smoking is predominately restricted to males (43). Estrogen was also posited to play a protective role against constituents within cigarette smoke, which encompasses genotoxic agents like HAAs, providing support and some emerging hypotheses underlying the sex-specific associations observed in Phase 2 (53,54).

8.3.4 Methodological Considerations

As discussed for Phase 1, the interpretation of epidemiologic evidence requires consideration of bias and confounding to weigh the main findings of an observational study in light of its strengths and limitations.

i. Temporality

In Phase 2, dietary intakes of HAAs and exposure to meat mutgenicity in the past year were assessed in relation to colorectal adenoma prevalence. A study of repeatability of dietary data over a seven-year period concluded that dietary patterns do not substantially change over time (55). Thus, the cross-sectional assessment of dietary intake of HAAs and meat mutagenicity was theorized to represent usual intakes within the biologically relevant time window prior to adenoma development (hypothesized to exist five to ten years prior to the date of screening colonoscopy). Temporal bias could hypothetically affect the results of Phase 2 if symptoms of colorectal carcinogenesis affected patterns of meat consumption and cooking habits in the past year. In Phase 2, a large proportion of participants were undergoing a first-time

colonoscopy screen; the reasons for participation included a first-degree family relative with a history of CRC and/or a positive fecal occult blood test. Those who were diagnosed with CRC at the time of their colonoscopy were excluded from Phase 2 due to the possibility of temporal bias. On the whole, the development of a colorectal adenoma is not usually associated with symptoms and thus, the assumption was made that dietary intakes during the previous year would not be affected by the disease process and would reflect adult dietary patterns in the last five to ten years reducing the potential for temporal bias.

ii. Selection Bias

Participants of Phase 2 were selected from consenting patients in the larger Canadian Cancer Society-funded study investigating the role of DNA methylation in colorectal adenomas. Briefly, relatively healthy individuals undergoing a screening colonoscopy were being recruited; therefore, those who were eligible included people with a positive fecal occult blood test and/or a first degree relative diagnosed with CRC. A participation rate of 67% was obtained for this larger study. Family history of CRC, age and sex were potential selection factors that have been related to the risk of developing colorectal adenomas and may be associated with dietary HAA intakes and/or meat mutagenicity exposure; however, these factors are antecedents of both the exposure and outcome and so could be controlled via multivariable analysis. Thus, it is unlikely that selection bias may have influenced the findings of Phase 2.

iii. Information Bias

In Phase 2, dietary exposures to HAAs and meat mutagenicity were derived using the CHARRED mutagen database as described for Phase 1 with the exception that the Phase 2 meat consumption module included fewer meat items, did not utilize colour photographs to standardize doneness level and serving size, and did not collect information on serving size.

However, exposure assessment was conducted prior to knowledge of colonoscopy findings; thus, though misclassification of exposure is more likely to occur in Phase 2 than in Phase 1, misclassification would be non-differential and result in biases towards the null in dichotomous representations (22).

One of the main strengths of Phase 2 was the inclusion of colonoscopy-confirmed colorectal adenoma status for all participants. However, the use of colonoscopy findings still has the potential for outcome misclassification. Specifically, miss rates for colonoscopies are reported to be between 15 to 25% for adenomas less than 5 mm; and from 0 to 6% for adenomas greater than 10 mm (59). Since adenomas that are smaller are more likely to be missed, the inclusion of small adenomas within the non-event group would introduce non-differential misclassification and bias the effect estimates towards the null. For Phase 2, the miss rate was expected to be lower than the published literature since only experienced physicians were performing the colonoscopies for the larger study and participants for whom the bowel was not sufficiently cleansed (which would reduce the physician's ability to detect an abnormality) were excluded from the larger study.

In Phase 2, newly diagnosed colorectal adenoma was the outcome of interest, however adenomas evolve subclinically for many years and, thus the detection of an adenoma at a colonoscopy is presumed to represent a mix of incident and prevalent cases. Incidence-prevalence bias or, the use of prevalent cases in a study, may have affected the interpretation of study results since prevalence depends on both the incidence and duration of disease after onset. This added nuance of duration, while affecting the prevalence of disease, may have influenced the interpretation of findings since increasing intake of dietary HAAs or meat mutagenicity exposure and genetic susceptibility may have either contributed to the risk of developing a

colorectal adenoma or the duration of having an adenoma which progressed slowly to CRC (that is, a less aggressive disease). Biologically, there is no evidence to support that exposure to dietary HAAs could actually slow the progression of colorectal carcinogenesis.

The functional effect of polymorphisms under investigation in Phase 2 was supported by mechanistic evidence. However, *a priori*, heterozygotes were grouped with the homozygous variant category to maximize statistical efficiency particularly for the investigations of gene-diet interactions. Therefore, it is recognized that misclassification may have been introduced if heterozygotes behaved more like the homozygous wild-type which would obscure an association if one existed.

iv. Confounding

A major strength of Phase 2 is the assessment of numerous potential determinants of colorectal adenoma and CRC risk. In addition, a strategy was developed to select the most appropriate variable representation for continuous covariates. Confounder selection was conducted by building a parsimonious model predicting colorectal adenoma risk. Relationships between dietary HAAs and covariates were evaluated to ensure that the parsimonious modelling strategy would capture predictors of outcome that were also associated with exposure. Finally, a sensitivity analysis was conducted to evaluate the impact of the confounder selection approach used by contrasting results obtained using a parsimonious modelling strategy versus a change-in-estimate approach.

The consideration of variable representation for continuous covariates was based on two priorities. First, the covariates of interest were selected based on their established relationship with adenoma risk; thus, comparability with the established literature, where the utilization of categorical representations was most common, was a priority. Second, if a linear relationship

was observed between a covariate and adenoma risk, use of continuous representation was prioritized since it would provide an appropriate representation and minimize the number of parameters for model building. Linear relationships were identified by a qualitative evaluation of the dose-response pattern of covariates represented categorically as well as the assessment of linear trend across categories. It was recognized that a small p-value obtained from a trend test does not equate that the association observed between a covariate and outcome is linear. Thus, in the event that a continuous covariate was erroneously represented, residual confounding may have occurred.

In Phase 2, sex, smoking status, previous diagnosis of irritable bowel syndrome, fruit and vegetable intake, dietary protein intake and biomarker levels of albumin, folate, vitamin B_{12} and lipid ratio were found to be predictors of colorectal adenoma at a 0.20 level of significance. All covariates have previously been shown to affect colorectal adenoma risk. In multivariable analysis, all predictors with the exception of previous diagnosis of irritable bowel syndrome, serum vitamin B_{12} and lipid ratio were found to significantly predict colorectal adenoma risk (at a p-value of 0.05).

To ensure that the parsimonious modelling strategy had captured the predictors of colorectal adenoma that were also associated with dietary HAAs and meat mutagenicity, bivariate analysis was conducted to assess covariate associations with dietary HAAs and meat mutagenicity. Bivariate analysis revealed strong associations between dietary HAAs, and BMI and lipid ratio; for meat mutagenicity, bivariate associations with BMI and lipid ratio were also observed. Strong correlations between dietary PhIP, MeIQx, DiMeIQx and meat mutagenicity was also found; but, confounding could not be assessed due to the strong collinearity within HAA exposure measures. The strong associations observed between specific dietary HAAs and

meat mutagenicity, and their ability to jointly predict colorectal adenoma risk could have been a source for residual confounding. In addition to the bivariate associations observed with BMI and lipid ratio the following relationships were observed between: 1) dietary PhIP, and fruit and vegetable intake; 2) dietary MeIQx, and smoking status or exposure to environmental tobacco smoke; 3) dietary DiMeIQx, and ethnicity or smoking status and; 4) meat mutagenticity, and dietary protein intake or age. Most of these covariates with the exception of environmental tobacco smoke and age were captured in the parsimonious model predicting colorectal adenomas. Age is a determinant of both colorectal adenoma and CRC risk in the published literature. However, due to the timeframe of exposure representation under investigation in the larger ongoing study, only a limited age range was represented which may have restricted the ability of age to predict colorectal adenoma risk and thus, confound the main relationships under investigation. There is some evidence to support the role of exposure to environmental tobacco smoke and CRC risk; however, only 24% of the study population in Phase 2 were exposed to environmental tobacco smoke and the dichotomous representation of exposure may have limited the ability to assess this relationship.

A sensitivity analysis was conducted in Phase 2 to evaluate the impact of the confounder selection approach used by contrasting results obtained using a parsimonious approach versus a change-in-estimate strategy. For both the main effect analysis for PhIP and the gene-diet interactions between PhIP and polymorphisms in *CYP1B1* and *XPD* on colorectal adenoma risk, results were comparable between the two confounder selection approaches.

v. Statistical Power

The moderate sample size in Phase 2 may have limited the ability to detect gene-diet interactions, though efforts were made at the onset of the study to select polymorphisms of

interest based on prevalence, demonstrated functional evidence and some previous support for their role in colorectal carcinogenesis. Though significant interactions between dietary HAAs/meat mutagenicity and polymorphisms in *CYP1B1* and *XPD* on colorectal adenoma risk were observed, our study may had limited statistical power to investigate additional interactions of smaller magnitudes. In addition, as an additional analysis, Phase 2 observed strong interactions between sex and dietary exposure to HAAs, and sex and meat mutagenicity on colorectal adenoma risk. The sex-specific difference in risk may additionally vary according to genetic susceptibility; however, the size of the study sample precluded the ability to assess gene-diet interactions separately among males and females.

8.4 Methodological and Scientific Contributions of this Thesis

The frequent consumption of meats has been linked to an increased risk of CRC. HAA exposure is a potential causal factor given that epidemiologic studies have shown the highest CRC risk for individuals who consume meats cooked well-done and who harbor either elevated activities of enzymes that bioactivate HAAs or decreased activities of DNA repair proteins. However, the reported associations between dietary factors and genetic polymorphisms remain inconsistent, and the associations cannot confirm the relationship between specific chemical exposures and carcinogenesis.

This dissertation exemplified the tenets of molecular epidemiology by revisiting the meat consumption-colorectal adenoma relationship through an emphasis on dietary HAAs, a thorough examination of the modifying effects of genetic susceptibility and an investigation of bulky DNA adduct formation as a potential carcinogenic mechanism. Independently, both Phases had certain methodological limitations, but together they provide support for the joint contributions of

dietary HAAs and genetic susceptibility on colorectal adenoma development and highlight the formation of DNA adducts as a plausible carcinogenic mechanism of action for HAAs.

Phase 1 provided a preliminary cross-sectional evaluation of the independent and joint contributions of dietary exposure to HAAs and individual susceptibility to DNA adducts, which are biomarkers of the biologically effective dose of HAAs along the hypothesized HAA-CRC continuum. At first glance, the lack of a strong independent relationship between dietary HAAs and bulky DNA adduct levels may appear to provide only limited evidence to support that the formation of DNA adducts is an early intermediate step from HAA exposure in the pathway to carcinogenesis. However, the identification of gene-diet interactions between NAT1 polymorphisms and exposure to dietary HAAs suggests that genetically susceptible subgroups exist in the population and may be at greater risk of the carcinogenic potential of dietary HAAs. Established evidence exists to support the roles of DNA adducts in carcinogenesis. Future studies that build on the findings from Phase 1 may have important implications for prevention, by providing the necessary information to modify cooking practices to reduce dietary exposures to HAAs.

In Phase 2, the role of dietary HAA intake, meat mutagenicity exposure and genetic susceptibility on the development of colorectal adenomas was examined; no independent relationships were observed. However, interpretable gene-diet interactions were found where higher intakes of dietary HAAs or meat mutagenicity exposure increased colorectal adenoma risk among those with an expected greater ability to bioactivate HAAs and lower activity to repair DNA damage. The associations observed in Phase 2 are not inconsequential given the high prevalence of colorectal adenomas in the Canadian population. As well, the use of colorectal adenomas in Phase 2 as a surrogate end point to CRC has a number of merits and implications.

First, adenomas are the precursor to the vast majority of CRC and thus, the relationships observed in Phase 2 may be generalizable to CRC. Second, adenomas are less symptomatic than CRC and hence the cross-sectional measures of dietary HAAs and meat mutagenicity exposures may be less likely to be affected by the disease process and may be more representative of past exposures. Finally, the removal of adenomas can prevent future occurrences of CRC. Therefore, understanding the etiology of colorectal adenomas may have the ability to prevent future cases of CRC. The identification of variation in genetic susceptibility to dietary HAAs/meat mutagenicity and the subsequent risk of colorectal adenoma implicates a role for dietary HAA exposures in colorectal carcinogenesis.

The basic premise of this research was that a better understanding of the relationship between meat consumption and CRC in human populations can be gained by isolating the effects of potential causal factors such as HAAs. This PhD dissertation aimed to clarify the role of dietary exposure to HAAs on colorectal carcinogenesis, and demonstrated the importance of interaction between diet and genetic susceptibility in the development of colorectal adenomas, precursor lesions to the vast majority of CRCs. This research combined epidemiological and molecular biological approaches to provide a better understanding of the link between meat consumption and cancer risk. The focus on HAAs as the potential link between meat consumption and subsequent cancer risk may contribute to the design of effective prevention strategies as exposure to these carcinogens is modifiable.

8.5 Generalizability

In Phase 1, a biologic relationship between exposure to dietary HAAs, genetic susceptibility and bulky DNA adduct formation was found. Though the majority of participants in Phase 1 were White, leading to a genetically homogenous study population, the main known

genetic determinants of bulky DNA adducts were captured in this study. Thus, the results of Phase 1 should be generalizable to populations with a similar ethnic distribution and the biologic relationship of higher intakes of dietary HAAs and higher bulky DNA adducts among genetically susceptible populations should be generalizable to all ethnicities provided the relevant genetic factors are considered.

In Phase 2, an increased risk of colorectal adenoma associated with high dietary HAA or meat mutagenicity exposures were found among those with genetic polymorphisms that predispose towards a higher activity to bioactivate HAAs and lower DNA repair. For polymorphisms in CYP1A2 and XPC, genotype distributions were not found to be in Hardy-Weinberg equilibrium which may be a characteristic of the underlying screening cohort from which participants of Phase 2 were recruited. That is, in the study population of Phase 2 those with a family history of CRC were overly represented, as compared to the general population, and may have contributed to skewing the distribution of allele frequencies. Like Phase 1, the majority of participants were White which may limit the generalizability of Phase 2 results. Nevertheless, Phase 2 provided evidence to support that colorectal adenomas are determined in part through the interaction between diet and genetic predisposition; if the appropriate genetic factors are considered in populations with different ethnic distributions, it is likely that the findings of Phase 2 are generalizable. The majority of CRCs arise from preexisting adenomas, and CRC and adenomas share a similar set of risk factors; thus, the findings of Phase 2 are anticipated to be generalizable to CRCs that develop through the adenoma-carcinoma sequence (34).

8.6 Multiple Comparisons

It is argued that studies that generate a large number of statistical tests have a markedly greater probability of generating false-positive results due to random error. The statistical aspect of the multiple comparisons issue is summarized as "if n independent associations are examined for statistical significance, the probability that at least one of them will be found statistically significant is $1 - (1- \alpha)^n$, if all n of the individual null hypotheses are true." Thus, if n is large, the probably of some statistically significant findings increases as a result of chance (56).

The simplest and most frequently used multiple testing adjustment method is the Bonferroni procedure, which adjusts the α level by the number of tests conducted (k), where p-values smaller than α/k are accepted as statistically significant (57). In the gene-diet interactions examined in Phase 1 and 2, effect modification by 9 and 10 genetic factors were investigated, respectively. For Phase 1, the adjusted α level of 0.006 would thus yield no significant interactions between any specific HAAs and genetic factors on bulky DNA adduct levels. For Phase 2, the adjusted α level of 0.005 applied to the genetic interactions investigated for PhIP would yield significant interactions with XPD rs13181 (p=0.004) and CYP1B1 rs10012 and rs1056827 (p=0.004) but not XPD rs1799793 (p=0.015). Though the Bonferroni method is a simple and widely applicable procedure, it has often been criticized for its application when the number of tests is large (n>5) and the correlations among the test statistics are low (57).

Rothman advocated that "the theoretical basis for advocating a routine adjustment for multiple comparisons involved the 'universal null hypothesis' that chance serves as the first-order explanation for observe phenomena" (56). The universal null hypothesis applied to Phase 1 and 2 would presume that all associations observed could only be due to random variation. In both Phase 1 and Phase 2, no adjustments were made to account for the multiple comparisons

involving the evaluation of the role of specific HAAs and genetic risk factors on bulky DNA adduct levels (Phase 1 outcome of interest) and colorectal adenoma risk (Phase 2 outcome of interest). These statistical comparisons were based on *a priori* hypotheses driven by experimental and epidemiologic evidence. Although the possibility of chance findings cannot be entirely excluded, the patterns of association observed in Phase 1 and 2 were consistent across genotype strata and previously reported associations; therefore the effect of chance related to multiple comparisons can not entirely explain the findings of this dissertation.

8.7 Changes from the Original Thesis Proposal

This dissertation originally was designed to examine the HAA-CRC association in two phases utilizing specific HAA-DNA adducts as biomarkers of exposure and early biologic effect. Specifically, in a cross-sectional study of 125 healthy volunteers, Phase 1 proposed to investigate the relationship between dietary exposure to HAAs and the formation of potentially carcinogenic HAA-DNA adducts in blood. For Phase 2, a cross-sectional study of 342 participants was funded to examine the relationship between specific HAA-DNA adducts measured in normal-appearing colon tissue, and the prevalence of colorectal adenoma. Phase 2 also proposed to determine whether adduct levels in blood were appropriate surrogates for adduct levels in colon tissue. The ultimate goal of the original thesis was to investigate key segments within the relationship between HAAs and CRC using biomarkers as tools to improve exposure assessment and contribute to the understanding of biologic mechanisms (Figure 8.1).

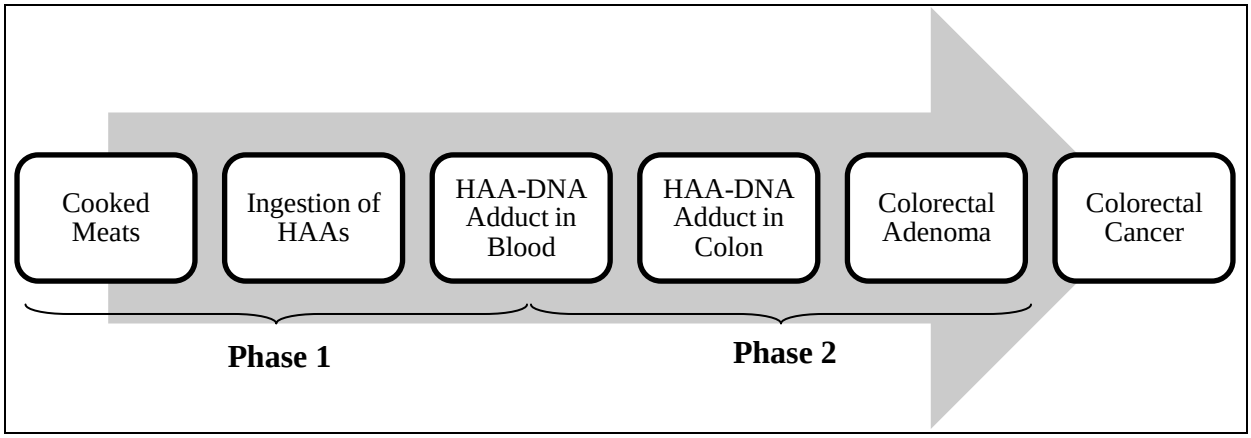

Figure 8.1: Conceptual model of proposed PhD dissertation.

The premise of this PhD dissertation hinged on the ability to quantify specific HAA-DNA adducts in human blood and tissue using a mass spectrometric approach. Through the receipt of operating grant funding for Phase 1 and 2, in collaboration with the Ontario Cancer Biomarker Network, over one year of time and resources was dedicated to assay development and pilot testing to identify and quantify specific HAA-DNA adducts in our participant samples. After repeated testing and development it was determined that a mass-spectrometric approach was not feasible to measure HAA-DNA adducts in leukocytic DNA obtained from a limited quantity of blood among healthy individuals (Appendix 1).

Due to this inability to measure specific HAA-DNA adducts, the ^{32}P-postlabelling method, was utilized for the quantification of total bulky DNA adducts. Phase 1 of this PhD dissertation was revised to assess the association between dietary intake of HAAs and bulky DNA adduct levels in blood in a cross-sectional study of 125 healthy volunteers. The major change from the original PhD objective for Phase 1 was the use of bulky DNA adducts as a surrogate measure for HAA-DNA adducts. Phase 2 of this research was revised to examine in a cross-sectional study of 342 participants undergoing a screening colonoscopy the relationships between: a) exposure to dietary HAAs and colorectal adenoma prevalence and; b) exposure to meat mutagenicity on the risk of developing colorectal adenomas. The major change from the

original PhD objective for Phase 2 was the absence of measures of HAA-DNA adducts in blood and colon tissue as biomarkers of exposure to HAAs.

8.8 Future Research Directions

In Phase 2, the quantification of bulky DNA adducts in blood leukocytes and normal-appearing colon tissue is ongoing. Future directions stemming from the results of this thesis include investigating: 1) the role of bulky DNA adduct levels measured in colon tissue on colorectal adenoma risk; 2) whether the formation of bulky DNA adducts is a mechanistic pathway through which HAAs contribute to colorectal carcinogenesis; 3) the use of blood leukocyte DNA adduct levels as a surrogate for colon tissue concentrations and; 4) whether polymorphisms in genes involved in the biotransformation of HAAs and DNA repair contribute to bulky DNA adduct levels in the colon.

Additionally, three research recommendations combining epidemiologic and toxicological approaches are highlighted to bridge research gaps and contribute to further establishing the association between HAAs and CRC risk at a molecular level.

i. Develop and validate biomarkers of HAA exposure

The development and validation of biomarkers of HAA exposure will improve exposure assessment and benefit future studies by providing evidence to support these biomarkers as unbiased estimators of dietary HAA exposure. HAA levels in hair and specific HAA-DNA adducts in blood lymphocytes and target tissues have the potential to serve as biomarkers of long-term exposures to dietary HAAs. Specifically, HAAs have been shown to have a high affinity for melanin-rich tissues like hair; due to the long half-life of HAAs in hair and the low invasive nature of obtaining hair samples, quantification of HAAs in hair may be a useful marker of long-term exposure to dietary HAAs.

In Phase 1, evidence of a strong relationship between dietary HAAs and bulky DNA adduct levels in blood leukocytes was not observed. The lack of an independent relationship is likely in part attributable to interactions with genetic susceptibility, as demonstrated by results from Phase 1, but may also be due either to the use of bulky DNA adducts as a surrogate measure for specific HAA-DNA adducts or the stability or suitability of blood leukocytes as the biologic matrix. The investigation of the relationship between dietary HAAs and specific HAA-DNA adducts, and the use of blood lymphocyte DNA adduct levels as surrogate measures for target site concentrations should be investigated in future studies. In addition, future validation studies should include the use of repeated measures to understand the stability and the relevant time window of exposure representation of HAA-DNA adducts. Finally, as demonstrated in Phase 1, future studies of biomarker development and validation will require the consideration of the role of individual susceptibility on the relationship between HAA exposures and HAA-DNA adduct levels. The proper planning and execution of a biomarker development and validation scheme will contribute to the understanding of the initial steps within the causal mechanism underlying dietary HAA exposure and cancer risk.

ii. Investigate the biologic mechanisms underlying the putative role of HAAs in carcinogenesis

Phase 2 of this research provided support for the role of dietary HAAs, exposure to meat mutagenicity, and genetic susceptibility in CRC etiology. Though established biologic evidence exists to support the formation of covalent adducts with DNA as a major route to which exposure to HAAs can increase the probability of carcinogenesis, future studies should investigate the association between specific HAA-DNA adduct formation at target sites like the colon mucosa and the risk of future development of colorectal adenoma and cancer.

Future studies should also encompass toxicological investigations to elucidate the mechanism through which polymorphisms in XPD affect the repair of bulky DNA adducts and/or HAA-derived DNA damage. Furthermore, the characterization of the mechanistic basis of how CYP1B1 and XPC polymorphisms contribute to the carcinogenic process is also of interest. Finally, future studies should consider additional biologic mechanisms through which HAAs may contribute to cancer etiology; specifically, evidence is emerging on the role of genotoxic compounds like HAAs in other pathways relevant to carcinogenesis like epigenetics (58).

iii. Strengthen the causal evidence for the role of HAAs in colorectal carcinogenesis

Future studies should focus on identifying and clarifying the interactions between dietary exposure to HAAs and additional genes involved in the biotransformation of HAAs and DNA repair on colorectal carcinogenesis. As well, as seen in the additional analysis conducted in Phase 2, the associations between dietary HAAs/meat mutagenicity and colorectal adenoma risk may differ by sex. Future studies should incorporate the consideration of sex-specific differences in the analysis of main effects as well as gene-diet interactions. Furthermore, studies should be designed and adequately powered to investigate potential gene-gene interactions. Finally, the establishment of the temporal sequence and the demonstration of dose-response effects between exposure to HAAs and subsequent CRC risk are key issues for future studies. Well-conducted studies that merge environmental and molecular epidemiologic approaches to longitudinally assess the association between exposure to HAAs, changes in intermediate endpoints, genetic susceptibility and future development of CRC, will strengthen the causal evidence for the role of HAAs in colorectal carcinogenesis

8.9 Conclusions

Meat consumption is associated with an elevated risk of CRC. Exposure to HAAs is one hypothesized explanation for this relationship. Difficulties with measuring HAA exposures accurately and reliably using self-reported dietary questionnaire data and individual genetic susceptibilities to these exposures may have contributed to the inconsistent evidence on the HAA-cancer relationship. This dissertation combined epidemiological and molecular biological approaches to address some of these issues.

Results of Phase 1 support that levels of bulky DNA adducts in blood leukocytes are a function of dietary exposure to HAAs and genetic susceptibility. However, the lack of a strong independent association between HAAs and bulky DNA adducts suggests that dietary HAAs are not a large contributor to bulky DNA adducts. Future research to clarify the potential of DNA adducts as biomarkers of HAA exposure should incorporate the quantification of specific HAA-DNA adducts, the use of repeated measures and contrast the use of different biologic matrices for biomarker measurement. Phase 2 of this research reaffirmed that dietary exposures to HAAs/meat mutagenicity and genetic susceptibility jointly contribute to the development of colorectal adenoma. Due to the high prevalence of colorectal adenoma in the general population, the results of Phase 2 are not inconsequential. The correlations between HAA exposures and the use of high temperature cooking methods or a preference for higher doneness levels are well-established; thus, changes in meat preparation practices may be more successful as a public health message for reducing colorectal adenoma and CRC incidence than previous recommendations for general reduction of meat in the diet.

Taken together, this dissertation highlighted the importance of individual susceptibility in evaluating the role of environmental exposures in carcinogenesis and ultimately, contributed to

an understanding of the role of HAAs as causative agents potentially underlying the link between meat consumption and CRC risk.

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APPENDIX 1: Method Development and Validation of a Mass-Spectrometric Approach for Quantification of Specific HAA-DNA Adducts in Human Blood Leukocytes

Exposure to heterocyclic aromatic amines (HAAs) is one hypothesized explanation for the meat-colorectal cancer (CRC) relationship. Difficulties with measuring HAA exposures accurately and reliably using self-reported dietary questionnaire data have likely contributed to the conflicting evidence on the HAA-CRC relationship. The original objective of this dissertation was to identify and quantify specific heterocyclic aromatic amine (HAA)-DNA adducts using mass spectrometry (MS) and investigate their role in colorectal carcinogenesis.

MS is an analytical technique that can be used to identify and quantify the amount of a specific compound (e.g. PhIP-DNA and MeIQx-DNA adducts) in a complex biologic matrix (blood or tissue) (1). To date, no research has been reported on characterization of the association between specific dietary HAAs, HAA-DNA adduct formation (measured in blood or colon tissue) and colorectal cancer risk. Until recently, the absence of analytical methods with sufficient specificity and sensitivity has hindered the quantification of specific HAA-DNA adduct concentrations. In 2008, Neale et al. published methods for HAA-DNA adduct analysis by MS, and in 2010 reported measurement of PhIP-DNA adducts in human saliva (2,3). Both publications supported the feasibility of applying the quantification of specific HAA-DNA adducts to population-based studies. Measurement of specific HAA-DNA adducts would provide an independent and unbiased confirmation of the role of HAAs in carcinogenesis and offer insights regarding the formation of DNA adducts as the mechanism leading to the malignant phenotype.

Through the receipt of operating grant funding for Phase 1 and 2, in collaboration with the Ontario Cancer Biomarker Network, over one year of time and resources were dedicated to

MS assay development and pilot testing to identify and quantify specific HAA-DNA adducts in our participant samples. After repeated testing and development, it was determined that a mass-spectrometric approach was not feasible for quantification of HAA-DNA adducts in our collected human samples. A summary of the method development and validation attempts for the detection and quantification of PhIP-DNA adducts is presented below.

1. Biomarker Method Development and Validation

Biomarker method development and validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of a biomarker in a given biological matrix is valid and reliable (4). The fundamental parameters for the development and validation of MS for the quantification of HAA-DNA adduct included consideration of validity and reliability (4).

i. Validity or accuracy refers to the extent to which the measurement represents the true value (concentration) of the biomarker of interest (5).

ii. Reliability describes the agreement between a series of measurements obtained when the procedure is applied repeatedly (4). A reliable assay determines the precision or the amount of random error associated with a measure.

2. Overview of the Quantification of PhIP-DNA Adducts using Mass Spectrometry

A selected reaction monitoring mass spectrometric (SRM-MS) approach was used for specific PhIP-DNA adduct identification and quantification. Figure 1 outlines an overview of the procedure for the detection and quantification of PhIP-DNA adducts using SRM-MS. Briefly, prior to SRM-MS, isolated DNA must be enzymatically digested to yield a mixture of natural (non-adducted) and adducted nucleosides (2). The digested sample was then cleaned up to remove the enzymes which may have created possible interferences with MS. SRM-MS

involved an initial separation phase whereby the DNA adduct of interest was selected (based on mass) from the digested DNA sample. The selected DNA adduct of interest was then fragmented and a unique molecular fragment pattern, representative of the adduct of interest, produced a signal. The signal represented the amount of the DNA adduct of interest (an unknown quantity) in the original digested DNA sample (6,7).

To determine the quantity of the DNA adduct of interest, a synthetic, stable isotope-labelled internal standard (IS) was added to the original DNA sample prior to enzymatic digestion. The IS used in this research was synthetically produced PhIP-DNA adducts that had been labelled with deuterium. These synthetic adducts were identical in every respect to the PhIP-DNA adduct of interestexcept in mass (due to the isotope label) and hence, could be differentiated by SRM-MS. Since the amount of the IS was known, the amount of the PhIP-DNA adduct could be measured by comparing the signals from the PhIP-DNA adduct (an unknown quantity) to the labelled IS (a known quantity). The IS was also used to account for any sample losses incurred during sample preparation and the variability of the SRM-MS assay.

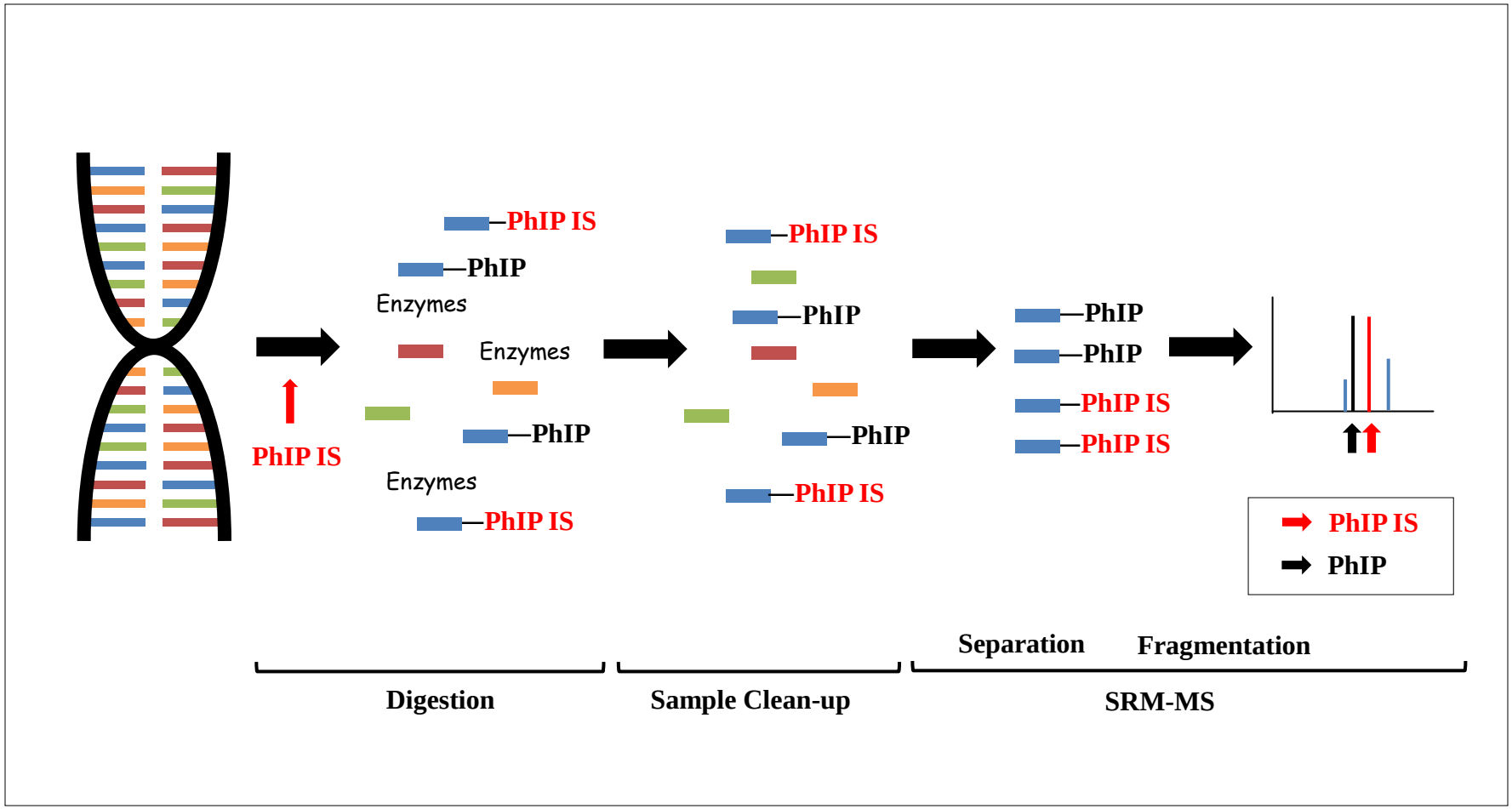

Figure 1. Detection and quantification of HAA-DNA adducts using MS; internal standard is abbreviated as IS.

3. Validity and Reliability of the Mass Spectrometry Method Development

The main aspects of the assay that required method development and optimization included DNA digestion, sample clean-up procedures and SRM-MS. Method development was initiated with the optimization of SRM-MS, followed by selection of the sample clean-up procedures and then finally optimization of DNA digestion. Two different types of test samples were generated for method development: one that included only the PhIP IS (Type A test sample) and another that included the PhIP IS and test sample DNA (Type B test sample). In the method development stage, test sample DNA was isolated from blood obtained from three volunteers under the same conditions of the sample collection and processing procedures utilized in Phases 1 and 2.

Type A test samples were used for initial set-up of the SRM-MS procedure and for measuring the validity of the assay; specifically, the recoveries of IS carried through different DNA digestion conditions and sample clean-up procedures were used to select the most optimal set of sample processing conditions for ensuring validity. Type B test samples were necessary to provide the appropriate biologic matrix in which to replicate the selected sets of conditions determined by Type A test samples. Recoveries of IS in Type B test samples were also used to determine validity as well as the reliability of the assay; reliability was assessed by calculation of the coefficient of variation of replicates processed under the optimal set of sample processing conditions.

i. Selected Reaction Monitoring Mass Spectrometry

Method development began with the optimization of the SRM-MS procedure for detection of specific PhIP-DNA adducts. This series of optimization was undertaken by the Ontario Cancer Biomarker Network using the recent published procedure by Neale et al., as a

reference (2). Optimization was conducted using the Type A test sample; based on replication of the fragmentation patterns of Neale et al., establishment of an acceptable lower limit of quantification and demonstration of linear range, the procedure was determined to be optimized.

ii. Sample Clean-up

After the enzymatic digestion of DNA, sample clean-up was necessary to remove contaminants and enzymes which may have interfered with the mass spectrometric detection of analytes of interest. Three sample clean-up procedures were tested; 10,000 nominal molecular weight limit centrifugal filters (Millipore, Bedford, MA), Zip Tip® Pipette Tips (Millipore, Bedford, MA) and PepClean™ C-18 Spin Columns (Pierce, Rockford, IL).

Selection of sample clean-up procedures was based on obtaining the highest recoveries from Type A test samples while balancing the ease for sample scale up. Both Zip Tips® and PepClean™ had high recoveries of IS (approximately 80%) but PepClean™ columns were more amendable than Zip Tips to scale up since up to 24 samples could be cleaned at once and thus, were selected. Using Type B test samples, a mean recovery of 77% was obtained with a coefficient of variation of 9%.

iii. DNA Digestion

Adequate digestion of DNA to yield a mixture of natural and adducted nucleosides was crucial to providing sensitive and specific DNA adduct structural information and thus greatly influenced the ability of MS to identify HAA-DNA adducts. In a study conducted by Neale et al., five digestion conditions were utilized to test the optimal conditions for DNA digestion and subsequent PhIP-DNA adduct detection using MS (2); conditions of each method are presented in Table 1.

Table 1. Conditions of DNA digestions using 85 to 150 µg of DNA

Method	Conditions
1	5 units of micrococcal nuclease and 0.01 units of spleen phosphodiesterase were added and incubated for 6 hours at 37˚C. 5 units of alkaline phosphatase were then added, and the samples were incubated overnight at 37˚C
2	5 units of nuclease P1 were added and incubated for 6 hours at 37˚C. 5 units of alkaline phosphatase were then added, and the samples were incubated overnight at 37˚C
3	10 units of DNase I were added and incubated for 1 hour at 37˚C. 5 units of nuclease P1 were added and incubated for 6 hours at 37˚C. 5 units of alkaline phosphatase were then added, and the samples were incubated overnight at 37˚C
4	10 units of DNase I were added and incubated for 1 hour at 37˚C. Then 0.01 units of spleen phosphodiesterase and 0.01 units of snake venom phosphodiesterase were added and incubated for 6 hours at 37˚C. 5 units of alkaline phosphatase were then added, and the samples were incubated overnight at 37˚C
5	10 units of DNase I were added and incubated for 1 hour at 37˚C. 5 units of micrococcal nuclease, 5 units of nuclease P1, 0.01 units of spleen phosphodiesterase and 0.01 units of snake venom phosphodiesterase were added and incubated for 6 hours at 37˚C. 5 units of alkaline phosphatase were then added, and the samples were incubated overnight at 37˚C

Utilizing Type A test samples, recoveries of IS were found to differ between different test conditions. Degradation or interference between the IS and the digestion enzymes was hypothesized to contribute to this sample loss in addition to losses incurred during sample clean-up and SRM-MS. It was found that Method 3 with slight modifications yielded the highest recoveries of IS. Modified Method 3 conditions included: the addition of 20 units of DNase I to test sample DNA and IS; the mixture was incubated at 37ºC for 1.5 hours. 5 units of nuclease P1 were then added and incubated at 37ºC for 6 hours followed by the addition of 5 units of alkaline phosphatase, and incubation at 37ºC for 18 hours.

At this stage of method development, two different Type B test samples (Type B test sample-1 and B-2) were utilized and 12 samples of each were analyzed under the optimal set of conditions (modified digestion Test 3 and PepClean^{TM} sample clean-up). For Type B test sample-1, 6 of 12 were successfully analyzed by MS. Recoveries of the IS ranged from 40 to 75% with a mean of 55% and standard deviation of 6.5; the coefficient of variation was 12%.

For Type B test sample-2, 7 of 12 were successfully analyzed by MS. Recoveries ranged from 50 to 100% with a mean of 75% and standard deviation of 6.1; the coefficient of variation was calculated to be 8%.

4. Application of Mass Spectrometry Method in Participant Samples

Throughout the MS method development, numerous attempts were made to scale up the analysis (to greater than 6 samples per day) which consistently led to a high percentage of sample failures (>50%). In the event of sample failures, the application of a biomarker assay to population-based studies with limited biological material would equate to a loss of study subjects and statistical power. Thus, an additional component that was critical for the application of the MS method to Phase 1 and 2 included having a failure rate of less than 10%.

The identification and quantification of PhIP-DNA adducts in Phase 1 participant samples were utilized in this final assessment of failure rate and to determine the applicability of MS in measuring PhIP-DNA adducts in Phase 1 participant samples. Seven participants were selected from Phase 1 based on representing low, medium and high dietary exposures to PhIP. Samples were analyzed in duplicates yielding a total of 14 test samples. First, 5 of 14 (36%) samples were successfully analyzed and had detectable but not quantifiable levels of PhIP-DNA adducts, 8 (57%) samples had undetectable levels of PhIP-DNA adducts and one sample failed (failure rate of 7%).

5. Conclusion

Taken altogether, the high percentage of sample failures throughout the development process and the inability to detect PhIP-DNA adducts in a large majority of participant samples from Phase 1 led to the change in methodology to measure DNA adducts. In place of specific

HAA-DNA adduct measures, this dissertation utilized the ^{32}P-postlabelling method to quantify bulky DNA adduct levels as a proxy for HAA-DNA adduct levels.

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APPENDIX 2: Phase 1 Study Questionnaire

Study ID

RESEARCH QUESTIONNAIRE

A research study of dietary factors and cancer risk

This questionnaire is part of a research study to help us learn more about whether important biologic changes in blood are useful tools for estimating dietary intake levels of potential cancer-causing compounds.

You do not have to answer any questions that make you feel uncomfortable. The answers that you provide will remain strictly confidential.

You will be identified by a study ID number and files containing your answers will be kept confidential. Your honesty is important for the success of this research. Answer each question as best you can – estimate if you aren't sure.

Thank you very much for your participation in this study.

Please check the boxes ☑ where choices are given and write in your answers where space is provided.

1. What gender are you? ☐ Female ☐ Male

2. What is your current age? _____ years

3. How would you best describe **your grandparents'** race, ethnicity or colour? Please specify as many as applicable:

Race, ethnicity or colour	**Your Maternal Grandmother (Mother's mother)**	**Your Maternal Grandfather (Mother's father)**	**Your Paternal Grandmother (Father's mother)**	**Your Paternal Grandfather (Father's father)**
White	☐	☐	☐	☐
Black	☐	☐	☐	☐
First Nations, Native or Aboriginal peoples of North America	☐	☐	☐	☐
Chinese	☐	☐	☐	☐
Japanese	☐	☐	☐	☐
Filipino	☐	☐	☐	☐
Korean	☐	☐	☐	☐
South Asian (e.g. East Indian, Pakistani, Punjabi, Sri Lankan)	☐	☐	☐	☐
South East Asian (e.g. Cambodian, Indonesian, Laotian, Vietnamese)	☐	☐	☐	☐
Arab/West Asian (e.g. Armenian, Egyptian, Iranian, Lebanese, Moroccan)	☐	☐	☐	☐
Latin American	☐	☐	☐	☐
Other *Specify:*_____	☐	☐	☐	☐

4. What is your current working situation? (C*heck all that apply)*
   - ☐ Unemployed
   - ☐ Retired
   - ☐ Homemaker, raising children, care of others
   - ☐ Student
   - ☐ Disabled, unable to work
   - ☐ Employed
     - ➡ a. In what kind of business, industry or service do you work? *(For example: road maintenance, shoe store, secondary school, municipal government, hospital etc.)*

       _____

     - b. What kind of work do you do? *(For example: nurse, researcher, teacher, factory worker, forestry technician, cook, etc.)*

       _____

The following 2 questions are related to your primary residence (the residence where you spend the majority of your time). This information is very important and will only be used for the purposes of this research study. Please be assured that, like all other information you have provided, these answers will be kept strictly confidential.

5. What is the street address of your primary residence (the residence where you spend the majority of your time)?

_____

Street Number *Street Name* *Apt/Unit Number*

_____

City *Postal Code*

6. How long have you lived at this address?

   _____

The next questions are about cigarette smoking.

7. During the **past 30 days** have you smoked at least 1 cigarette?
   - ☐ No *(Go to Question 8)*
   - ☐ Yes
     - ➡ a. In the past 30 days, on how many days did you smoke at least 1 cigarette?
       ____ days
     - b. On the days that you smoked, **on average**, how many cigarettes did you usually smoke in **one day**?
       ___ cigarettes per day

8. Have you ever smoked at least 1 cigarette a day for **12 months or more**?
   - ☐ No *(Go to Question 9)*
   - ☐ Yes
     - ➡ a. How old were you when you started smoking?
       ___ years old
     - b. When you smoked the most, how many cigarettes did you usually smoke in one day?
       ___ cigarettes per day
     - c. Are you still smoking?
       - ☐ No ➡ How old were you when you stopped smoking?
         ___ years old
       - ☐ Yes

9. During the **past 30 days** have you smoked cigars, cigarillos, bidis, a pipe, or used snuff or chewing tobacco?
   - ☐ No
   - ☐ Yes

Second-hand smoke exposure happens when you are close enough to someone who is smoking to smell the tobacco smoke.

10. During the **past 30 days**, on average, how many **hours per week** were you exposed to second hand smoke?
    - ☐ 0 or less than 1 hour
    - ☐ 1-4 hours
    - ☐ 5-9 hours
    - ☐ 10-14 hours
    - ☐ 15 or more hours

The next questions ask about the amount of time you spend in or near traffic.

11. During a **typical week**, how much time do you spend driving on roads (*include time spent as a driver or passenger in a car, bus, motorcycle, or other motorized vehicle*)?

    ____ hours per week

12. During a **typical week**, how much time do you usually spend involved in activities (other than driving or riding in a motorized vehicle) within 200 metres (length of 2 football fields) of a busy street or major road?
    (For example: walking, running, biking, etc.)

    ____ hours per week

The next questions ask about your home.

13. What heating system(s) is used to heat your home? *(Check all that apply)*
    - ☐ Steam or hot water system, including boiler
    - ☐ Forced air furnace
    - ☐ Heating stove (including wood stove)
    - ☐ Electric heat (including electric baseboard heaters)
    - ☐ Other ➡ *Specify:*_____

14. What source(s) of fuel is used to heat your home?*(Check all that apply)*
    - ☐ Oil or other liquid fuel
    - ☐ Natural gas (piped gas)
    - ☐ Propane (bottled gas)
    - ☐ Electricity
    - ☐ Wood
    - ☐ Other ➡ *Specify*:_____

The next questions ask about how often you eat certain foods. For Questions 15-23, please answer how often you eat these foods either each day *or* each week *or* each month.

15. **Over the last 12 months**, **on average**, *how many* times did you drink fruit juices (with no sugar or sweetener added) such as orange, grapefruit or tomato juice?

    _____ times per day ***or***
    _____ times per week ***or***
    _____ times per month

16. Not including fruit juices, **over the last 12 months**, **on average**, *how many* times did you eat fruit (including canned, dried, frozen and fresh fruit) on its own or with other food?

    _____ times per day ***or***
    _____ times per week ***or***
    _____ times per month

17. **Over the last 12 months**, **on average**, *how many* times did you eat a green salad (lettuce and leafy greens with or without other ingredients)?

    _____ times per day ***or***
    _____ times per week ***or***
    _____ times per month

18. **Over the last 12 months**, **on average**, *how many* times did you eat potatoes (**not** including french fries, other fried potatoes or potato chips, sweet potatoes or yams)?

    _____ times per day ***or***
    _____ times per week ***or***
    _____ times per month

19. **Over the last 12 months**, **on average**, *how many* times did you eat carrots (including canned, frozen or fresh) either on their own or with other food?

    _____ times per day ***or***
    _____ times per week ***or***
    _____ times per month

20. Not including carrots, potatoes or green salad, **over the last 12 months**, **on average** , *how many* times did you eat other vegetables?

    _____ times per day ***or***
    _____ times per week ***or***
    _____ times per month

21. **<u>Over the last 12 months</u>**, **on average**, *how many* times did you eat hot or cold breakfast cereals?

_____	times per day	***or***
_____	times per week	***or***
_____	times per month	

22. **<u>Over the last 12 months</u>**, **on average**, *how many* times did you eat whole grain products? *(Whole grain products include: 12-grain, multi-grain, whole wheat, rye, oatmeal, and pumpernickel breads used for toast, bagels, rolls and in sandwiches, whole grain crackers, whole grain cereals, whole grain pasta, etc.).*

_____	times per day	***or***
_____	times per week	***or***
_____	times per month	

23. During the **past 12 months** have you had at least one drink of beer, wine or liquor?
1 drink = 12oz beer (1 bottle), 5oz wine (1 glass) or 1.5oz hard liquor (spirits)
☐ No *(Go to Question 24)*
☐ Yes ➡ Indicate in the chart below how many drinks, **on average**, you consumed

per week over the past 12 months:

Type of Beverage	None	Less than 1 per week	1 to 7 per week	8 to 14 per week	15 to 21 per week	More than 21 per week
Beer (12 oz.)	☐	☐	☐	☐	☐	☐
Wine (5 oz.)	☐	☐	☐	☐	☐	☐
Liquor (1.5 oz.)	☐	☐	☐	☐	☐	☐
Coolers, breezers (12 oz.)	☐	☐	☐	☐	☐	☐

The next questions ask about how you cook your food.

24. What kind of stove do you **most often** use for cooking?
☐ Electric
☐ Natural gas
☐ Kerosene
☐ Propane
☐ Other gas
☐ Other ➡ *Specify:*_____

25. **During the past year**, how often did you or someone else cook food **inside your home** by grilling, frying or sautéing?

_____ times per day ***or***
_____ times per week ***or***
_____ times per month

26. Do you have a fan over your stove?
☐ No
☐ Yes
➡ a. When cooking, how often is the fan used?
☐ All the time
☐ Some of the time
☐ None of the time
b. Does the fan take fumes outside the home?
☐ No
☐ Yes

INSTRUCTIONS

Questions 27 - 38

In this section, you will be asked about your **typical** eating habits in the **past year** (divided into summer and winter months – starting with summer months) for meat, poultry and fish. You will be asked to check off the number of times you ate the food items, how they were prepared, the usual serving size and how well they were cooked (level of doneness). This includes meals prepared at home and eaten out. If these items were prepared differently than indicated, please include this under "other." Please try to give your most accurate estimate.

There are pictures provided to illustrate the serving size and doneness of meats. Also, to help you identify the methods of cooking, here are definitions for each of the cooking methods:

Method Name	Definition
Baked	Cooked in an oven with very little or no added liquid
Deep-fat fried	Cooked by immersing completely in hot fat
Grilled/Barbecued	Cooked over a charcoal or gas grill (inside or outside the home)
Oven-broiled	Cooked by direct exposure to the heat source in the oven
Pan-fried	Cooked in a small amount of hot fat in an open shallow pan
Stewed	Cooked by simmering in liquid in the oven, on the stovetop, or in a slow cooker

Please see the following **example** before you begin:
lease answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in all the appropriate boxes ☑.
If you make a mistake, please circle the correct answer ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Rare	Medium	Well-done
									Medium Serving Size: 4 oz or ¼ lb					
Hamburger, Cheeseburgers														
Pan-fried	☐	☑	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☑	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☑	☑	☐	☐	☐	☑	☐	☐	☐	☑
Other *Specify*: Microwave	☐	☐	☐	☑	☐	☐	☐	☐						

SUMMER MONTHS (APRIL – OCTOBER)

27. Please answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in all the appropriate boxes ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Rare	Medium	Well-done
									Medium Serving Size: 4 oz or ¼ lb					
Hamburger, Cheeseburgers														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Beef Steaks														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Roast Beef														
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Deep Fried Fish/Fish Sandwich														
Deep fat fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Rare	Medium	Well-done
									Medium Serving Size: 4 oz or ¼ lb					
Other Fish Except Deep-Fried Fish (including tuna)														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Casserole	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						

SUMMER MONTHS (APRIL – OCTOBER)

28. Please answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in the appropriate boxes ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Just-Done	Well-Done	Very Well-Done
									Medium Serving Size: 4 oz or ¼ lb					
Fried Chicken														
Deep fat fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			
Chicken eaten with skin (including on sandwiches)														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Stewed	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Chicken eaten without skin (including on sandwiches)														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Stewed	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Sandwich meat or cold cuts														
Any sandwich meats (such as luncheon meat, bologna, salami) excluding ham	☐	☐	☐	☐	☐	☐	☐	☐						

SUMMER MONTHS (APRIL – OCTOBER)

29. Please answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in the appropriate boxes ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Just-Done	Well-Done	Very Well-Done
									Medium Serving Size: 4 oz or ¼ lb					
Pork Chops														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify:*_____	☐	☐	☐	☐	☐	☐	☐	☐						
Roast Pork														
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify:*_____	☐	☐	☐	☐	☐	☐	☐	☐						
Ham Slice or Ham Steak														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other *Specify:*_____	☐	☐	☐	☐	☐	☐	☐	☐						

SUMMER MONTHS (APRIL – OCTOBER)

30. Please answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in the appropriate boxes ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Just-Done	Well-Done	Very Well-Done
Hot Dogs or Franks														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	Medium Serving Size: 1-2 hot dog			☐	☐	☐
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			
Other *Specify:*_____	☐	☐	☐	☐	☐	☐	☐	☐						
Bacon														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	Medium Serving Size: 2 strips			☐	☐	☐
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			
Other *Specify:*_____	☐	☐	☐	☐	☐	☐	☐	☐						
Sausage (including breakfast, Italian, Polish)														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	Medium Serving Size: 2 links or 2 patties			☐	☐	☐
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			
Other *Specify:*_____	☐	☐	☐	☐	☐	☐	☐	☐						
Gravy														
Made from meat drippings	☐	☐	☐	☐	☐	☐	☐	☐	Medium Serving Size: 2 Tbsp					
Store bought canned gravy	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify:*_____	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			

31. **<u>In the summer months *(April - October)*</u>, on average**, *how often* was oil, butter, margarine or other fat used to FRY, SAUTE, BASTE OR MARINATE any meat, poultry or fish that you ate? (*Exclude 'Pam'-type spray*)

- ☐ Less than once per month
- ☐ 1 time per month
- ☐ 2-3 times per month
- ☐ 1 time per week
- ☐ 2 times per week
- ☐ 3-4 times per week
- ☐ 5-6 times per week
- ☐ 1 time per day
- ☐ 2 or more times per day

32. **<u>In the summer months *(April - October)*</u>, on average**, what kind of fat is usually used for frying and sautéing meat, poultry or fish at home? (*Exclude 'Pam'-type spray*)

- ☐ Real butter
- ☐ Margarine
- ☐ Vegetable oil
- ☐ Vegetable shortening
- ☐ Lard
- ☐ None
- ☐ Other ➡ *Specify*:_____

WINTER MONTHS (NOVEMBER – MARCH)

33. Please answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in the appropriate boxes ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Rare	Medium	Well-done
									Medium Serving Size: 4 oz or ¼ lb					
Hamburger, Cheeseburgers														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify:*_____	☐	☐	☐	☐	☐	☐	☐	☐						
Beef Steaks														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Roast Beef														
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Deep Fried Fish/Fish Sandwich														
Deep fat fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Rare	Medium	Well-done
									Medium Serving Size: 4 oz or ¼ lb					
Other Fish Except Deep-Fried Fish (including tuna)														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Casserole	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						

WINTER MONTHS (NOVEMBER – MARCH)

34. Please answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in the appropriate boxes ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Just-Done	Well-Done	Very Well-Done
									Medium Serving Size: 4 oz or ¼ lb					
Fried Chicken														
Deep fat fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			
Chicken eaten with skin (including on sandwiches)														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Stewed	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Chicken eaten without skin (including on sandwiches)														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Stewed	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Sandwich meat or cold cuts														
Any sandwich meats (such as luncheon meat, bologna, salami) excluding ham	☐	☐	☐	☐	☐	☐	☐	☐						

WINTER MONTHS (NOVEMBER – MARCH)

35. Please answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in the appropriate boxes ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Just-Done	Well-Done	Very Well-Done
									Medium Serving Size: 4 oz or ¼ lb					
Pork Chops														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Roast Pork														
Baked	☐	☐	☐	☐	☐	☐	☐	☐						
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Ham Slice or Ham Steak														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						

WINTER MONTHS (NOVEMBER – MARCH)

36. Please answer the following questions about usual use, serving size and level of doneness for each food by putting a check mark in the appropriate boxes ☑.

	Usual Use								Usual Serving Size			Usual Level of Doneness		
	Never	Less than 1 per month	1-3 per month	1 per week	2-4 per week	5-6 per week	1 per day	2 or more per a day	Small	Medium	Large	Just-Done	Well-Done	Very Well-Done
Hot Dogs or Franks														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	Medium Serving Size: 1-2 hot dog			☐	☐	☐
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Bacon														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	Medium Serving Size: 2 strips			☐	☐	☐
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Sausage (including breakfast, Italian, Polish)														
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	Medium Serving Size: 2 links or 2 patties			☐	☐	☐
Grilled or barbequed	☐	☐	☐	☐	☐	☐	☐	☐						
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐						
Gravy														
Made from meat drippings	☐	☐	☐	☐	☐	☐	☐	☐	Medium Serving Size: 2 Tbsp					
Store bought canned gravy	☐	☐	☐	☐	☐	☐	☐	☐						
Other *Specify*:_____	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐			

37. **<u>In the winter months *(November - March)*</u>, on average**, *how often* was oil, butter, margarine or other fat used to FRY, SAUTE, BASTE OR MARINATE any meat, poultry or fish that you ate? (*Exclude 'Pam'-type spray*)

- ☐ Less than once per month
- ☐ 1 time per month
- ☐ 2-3 times per month
- ☐ 1 time per week
- ☐ 2 times per week
- ☐ 3-4 times per week
- ☐ 5-6 times per week
- ☐ 1 time per day
- ☐ 2 or more times per day

38. **<u>In the winter months *(November - March)*</u>, on average**, what kind of fat is usually used for frying and sautéing meat, poultry or fish at home? (*Exclude 'Pam'-type spray*)

- ☐ Real butter
- ☐ Margarine
- ☐ Vegetable oil
- ☐ Vegetable shortening
- ☐ Lard
- ☐ None
- ☐ Other ➡ *Specify*:_____

39. How has your use of the following foods **changed** over the **past 12 months**?

Fish ☐ Use has decreased ☐ Use about the same ☐ Use has increased	**Beef Steak** ☐ Use has decreased ☐ Use about the same ☐ Use has increased
Hamburger ☐ Use has decreased ☐ Use about the same ☐ Use has increased	**Pork** *(including ham, bacon and sausage)* ☐ Use has decreased ☐ Use about the same ☐ Use has increased
Chicken ☐ Use has decreased ☐ Use about the same ☐ Use has increased	

The next questions ask about your use of dietary supplements, medications and your medical history.

40. **Over the last 12 months**, have you taken any of the following types of supplements or preparations on a regular basis – that is, **more than once per week for at least 1 month during the past 12 months**? (*Check all that apply*)

Supplement or preparation	YES	NO
Fiber supplements such as Metamucil, Fiber-sure, Benefiber or other fiber supplements	☐	☐
Multi-vitamins (One-a-day, Spectrum, Centrum etc.)	☐	☐
Other supplements that are **not** part of a multi-vitamin ☐ NO ☐ YES ➡ *Check all that apply from the list below.*		
Vitamin A	☐	☐
Vitamin C	☐	☐
Vitamin D	☐	☐
Vitamin E	☐	☐
Zinc	☐	☐
Beta carotene	☐	☐
Calcium or calcium-containing antacid	☐	☐
Magnesium	☐	☐
Vitamin B6	☐	☐
Vitamin B12	☐	☐
Vitamin B-complex	☐	☐
Folic acid	☐	☐
Selenium	☐	☐
Iron (Palifer, Eurofer etc.)	☐	☐
Niacin	☐	☐
Other: *Specify*_____	☐	☐

41. During the **past 30 days** have you used any medicated creams or ointments to treat psoriasis or eczema (atopic dermatitis)?
    a. No *(Go to Question 46)*
    b. Yes
        ➡ a. How often did you use these medications?
            ___ times per week

        b. Please list the name(s) of the medicated creams/ointments you have used in the past 30 days:

        _____

        _____

42. Were you ever told by a family doctor/general practitioner or specialist that you had any of the following conditions or symptoms?

Medical Condition	YES	NO
Cancer: Type: _____	☐	☐
Angina (Coronary Artery Disease)	☐	☐
High blood pressure (hypertension)	☐	☐
Heart Attack (Myocardial Infarction)	☐	☐
Irritable bowel syndrome	☐	☐
Type 1 Diabetes	☐	☐
Type 2 Diabetes	☐	☐
Diabetes: Type unknown	☐	☐
Crohn's Disease	☐	☐
Ulcerative Colitis	☐	☐

Thank you very much for completing this questionnaire.

Questionnaire Guide – Meat Doneness Pictures

Please use these pictures to help estimate your usual level of doneness

Hamburger

Rare

Medium

Well-Done

Beef Steaks

Rare

Medium

Well-Done

Chicken

Just-Done

Well- Done

Very Well-Done

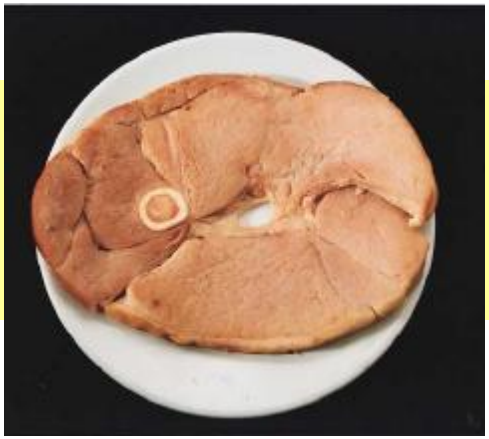
Pork Chops

Just-Done

Well- Done

Very Well-Done

Ham Slice



Just-Done

Well- Done

Very Well-Done

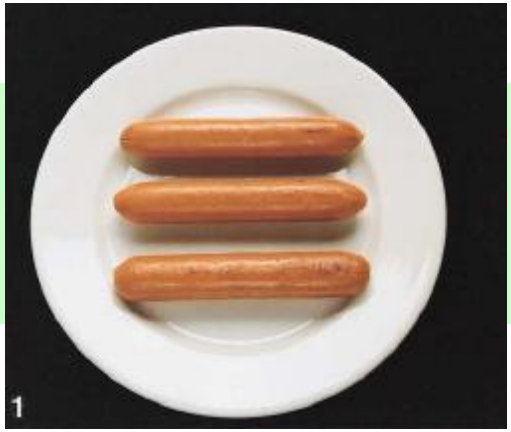
Bacon

Just-Done

Well- Done

Very Well-Done

Sausage, Hot Dogs or Franks



Just-Done

Well- Done

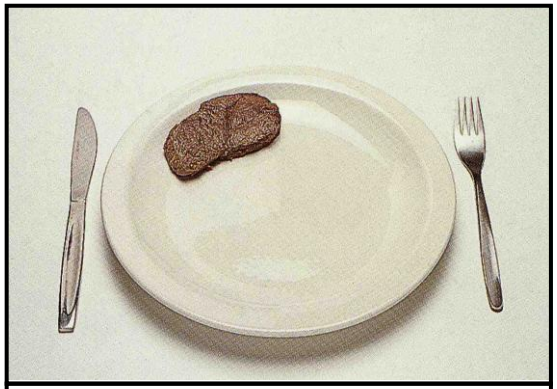
Very Well-Done

Questionnaire Guide – Serving Size Pictures

Please use these pictures to help estimate your usual serving sizes

- Medium serving sizes are given on the Questionnaire
- A small serving is about one-half (1/2) the medium serving size or less
- A large serving is about one and one-half (1 1/2) times the medium serving size or more

Steak/Chicken/Pork Chop:



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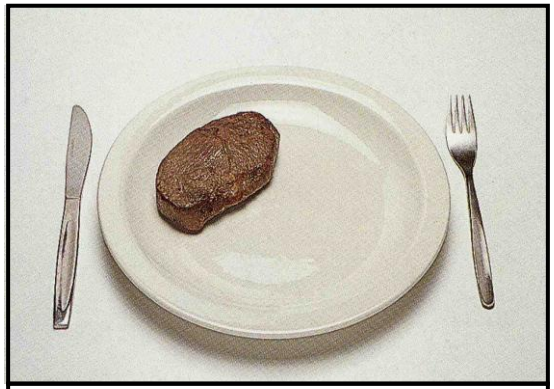

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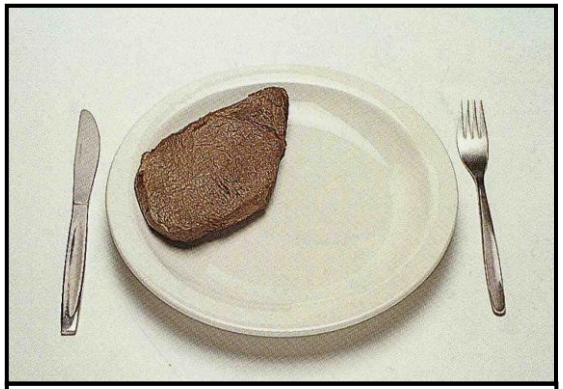
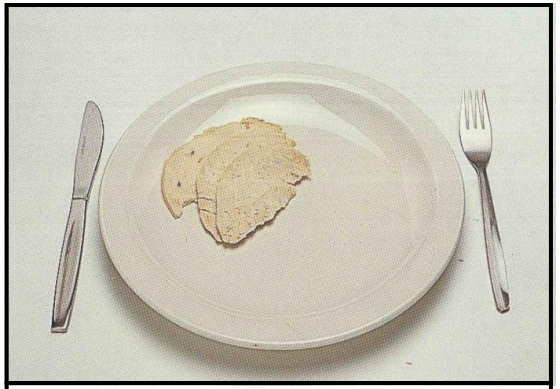


Large

Roast Beef/Roast Chicken:



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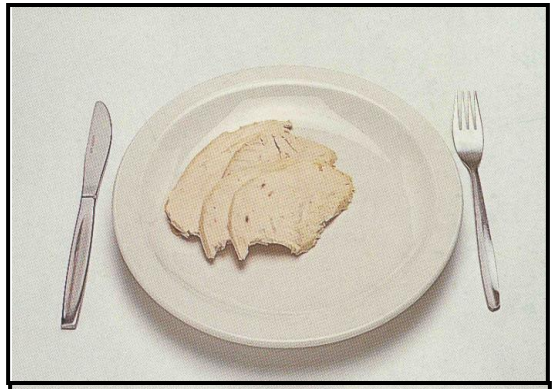

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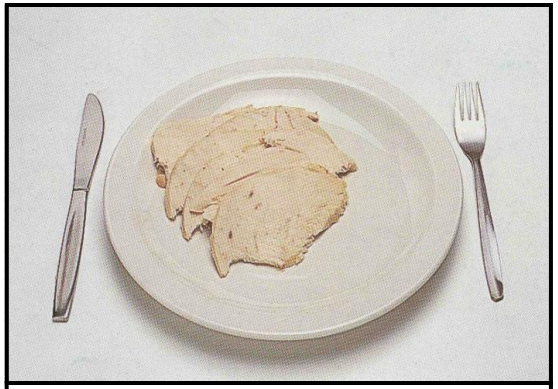
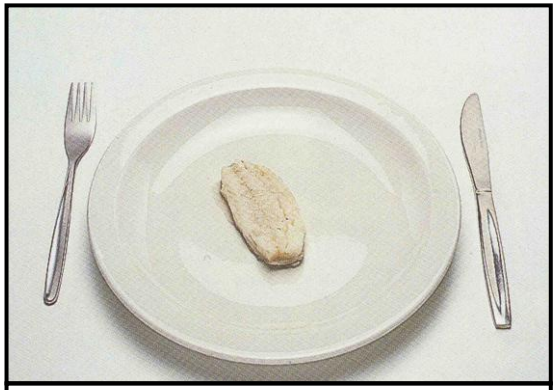


Large

Fish:



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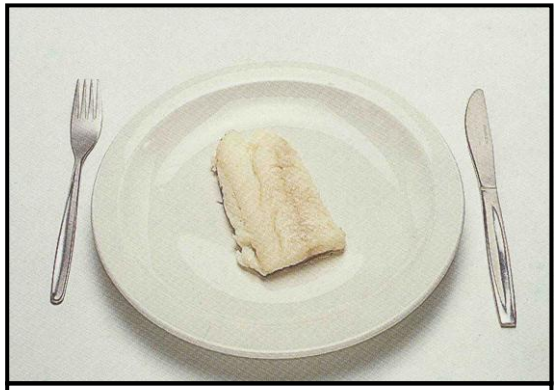

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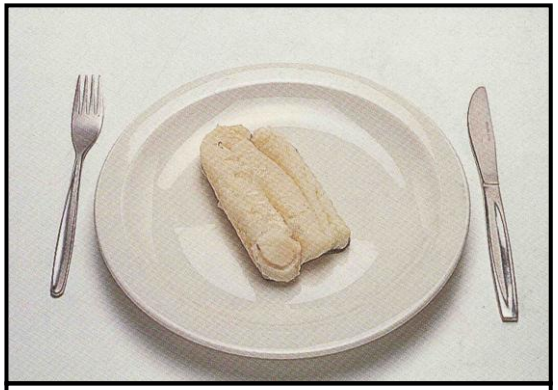
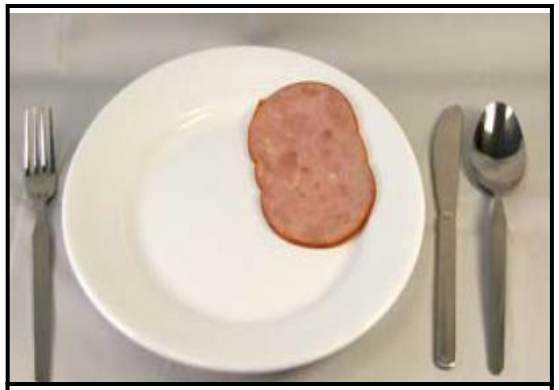
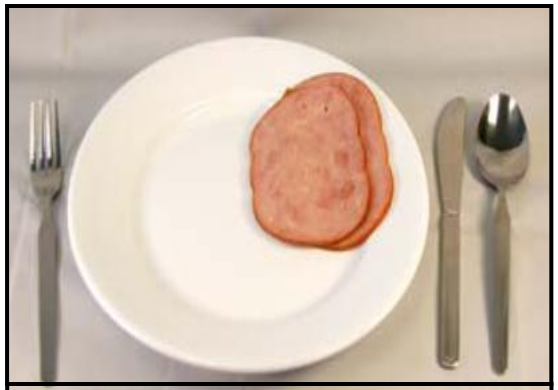


Large

Ham Slice:



Small



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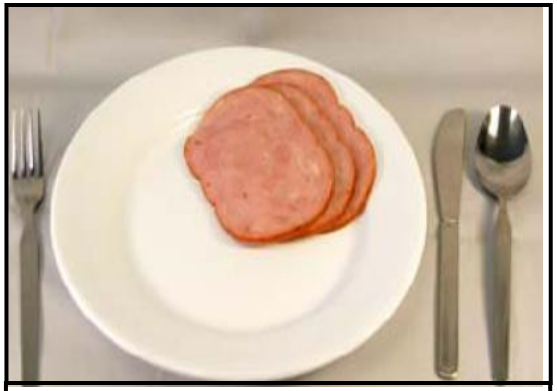


Large

APPENDIX 3: 32P-Postlabelling Assay for Bulky DNA Adducts

Adduct labeling and separation with 2-dimensional TLC

Solutions:

enzyme-mix:	number of samples	12	24
	pH 6.0		
	SoSuccinate 100 mM; CaCl2 5 mM	28.0 µl	52.8 µl
	MN (0.25 U/µl)	22.4	42.2
	SPD (2 µg/µl)	19.6	37.0
	Total	70	132

Note: enzyme-mix can be prepared in advance and stored at -20°C in small aliquots.

NP1-mix	NP1 (2.5 µg/µl)	35 µl	66 µl
	Sodium Acetate 0.25 M	21	39.6
	ZnCl2 0.3 mM	14	26.4
	Total	70	132

Labelmix	Kinase buffer*	21 µl	39.6 µl
	PNK (10 U/µl)	7	13.2
	[32P]-ATP (50 µCi/ sample)	X	X
	mQ-water	14-X	26.4-X
	Total	42	70

*Kinase Buffer: pH 9.5
0.2 M Bicine
0.1 M MgCl2
0.1 M DTT
10 mM Spermidine

Nuclease P1 enrichment procedure

- Dissolve the sample-DNA in 2 mM Tris, pH 7.4 at a concentration of 2 mg/ml, determined spectrophotometrically (make sure that the DNA is dissolved homogeneously).
- use 5 µl DNA of samples and standards. (± 10 µg)
- add 5 µl enzyme-mix, and incubate for 3 hours at 37°C.(Total volume = 10 µl; 5 µl for adductlabeling + 5µl for nucleotide quantitation.)
- add 5 µl NP1-mix to 5 µl DNA-digest.
- Remaining 5 µl digest is used for nucleotide-quantitation (see number 3.)
- incubate 40 minutes at 37°C
- Stop the NP1 reaction by adding 2.5 µl 0.5 M unbuffered Tris.
- Add 3 µl label mix (50 µCi / 3 µl) =>Total Volume: 15 .5 µl
- incubate 30 minutes at 37°C
- Spot 14 µl on a Machery-Nagel polyethyleneimine (PEI) cellulose-sheet (10x10 cm; origin (2,2)) with a Whatman paperwick (10x23 cm). Use rest (± 1.5 µl) for NP1-check and ATP excess check.
- Chromatography as shown in drawing.
- Quantitation adducts and background activity (we use a phosphor imaging technology, instead of autoradiography, but results are similar).

Solvent systems:
1: 1 M NaH2PO4, pH 6.5 (overnight on a Wick)
2: 5.3 M Lithium Formate, 8.5 M Urea, pH 3.5
3: 1.2 M LiCl, 0.5 M Tris, 8.5 M Urea, pH 8.0
4: 1.7 M NaH2PO4, pH 6.0 (overnight on a Wick) see figure

between each step, sheets are washed in demi-water for 5 minutes and 2 minutes (fresh water) and dried.

Butanol extraction method

- digest 5 µl DNA (2 µg/µl) as described above with SPD and MN.
- Use 5 µl of this digest and dilute with 25 µl 10 mM TBA (TetraButylAmmonium) and 220 µl Ammonium Formate (11.8 mM pH 3.5) to a total volume of 250 µl.
- extract twice with 250 µl water saturated 1-butanol (1 minute vortex, pool butanol fractions).
- extract the butanol 2 times with 1-butanol saturated water.
- add 1 µl 1 M Tris, pH 9.5
- dry samples in vacuo.
- redissolve dried extracts in 12.5 µl mQ and label the nucleotides according to the NP1-protocol as described above.

Nucleotide quantitation

solutions:

Label mix:	30	µl	Kinase Buffer
	6.3	µl	PNK (10 U/µl)
	30	µl	cold ATP 6mM (10-fold excess of ATP !!!)
	x	µl	[32P]-ATP (15 µCi/sample)
	83.7-x	µl	mQ
Total	150		

- dilute 3 µl of the DNA-digest with 85 µl Tris 5 mM pH 7.4
- Take 5 µl of this dilution and add 5 µl label mix => Total= 10µl
- incubate for 30 minutes at 37°C
- dilute with 750 µl mQ-water
- total volume 760 µl; spot 3 µl on a PEI-Sheet (Merck).

short overview:

3 µl	hydrolysed DNA
85 µl	Tris 5 mM pH 7.4
88 µl	Total

5 µl	diluted DNA-digest or a dAp standard (27.5 pmol / µl)
5 µl	label mix
10 µl	

750 µl	MQ-WATER

spot 5 µl on a PEI-sheet

Chromatography with 0.12 M NaH2PO4 , pH 6.8 on wick (10 X23 cm, same sheet as adducts, other side up)

*If the four spots of nucleotides are double, this implies a contamination with RNA, the samples must be discarded.

*If spots A and C are respectively more intens than T and G, this implies a deficit of ATP or poor labelling.

*a tailing spot at the origin is an indication of protein contamination.

Calculation of the DNA-concentration:

$$[DNA] = \frac{(\text{cpm sample dAp- background})}{(\text{cpm standard dAp- background})} \times 27.5 \times (100/29) \times (1/3240) \times (88/3) \times (10/5)$$

27.5:	dAp standard was 27.5 pmol/µl
100/29:	dAp = 29 % of total dNp
1/3240:	1 µg DNA= 3240 pmol dNp
88/3:	dilution with Tris
10/5:	5 µl sample + 5 µl enzyme mix.

Always include a water-blanc, because some enzymes / solutions might be contaminated with dNp. Use intensity of water-blanc as background.

NP1-Check and Check for ATP-excess

- 1.5 µl of the adduct labeling was nòt spotted; dilute this rest with 750 µl mQ-water
- spot 3 µl on a PEI-sheet and develop with 0.12 M NaH2PO4, pH 6.8
- autoradiography and analysis (qualitative, not quantitative)

Note: If ATP was in excess, you should find an ATP spot
If NP1 digested the normal nucleotides, no (little) dNp spots should be found.

Solvents for aromatic adducts

D1:
1.0 M NaH_2PO_4, pH 6.5
- dissolve 345 g $NaH_2PO_4.H_2O$ in about 2000 ml water
- adjust to pH 6.5 with 10 M NaOH, letting solution cool to room temperature before completing last part of titration
- make up to 2.5 litre with H_2O

Note: This solvent is just on the point of precipitation : raising the pH decreases the solubility of the salt, so accurate titration is important.

D2:
5.3 M Lithium formate
8.5 M Urea
pH 3.5
- This solvent takes 4 hours to make.
- prepare in fume hood

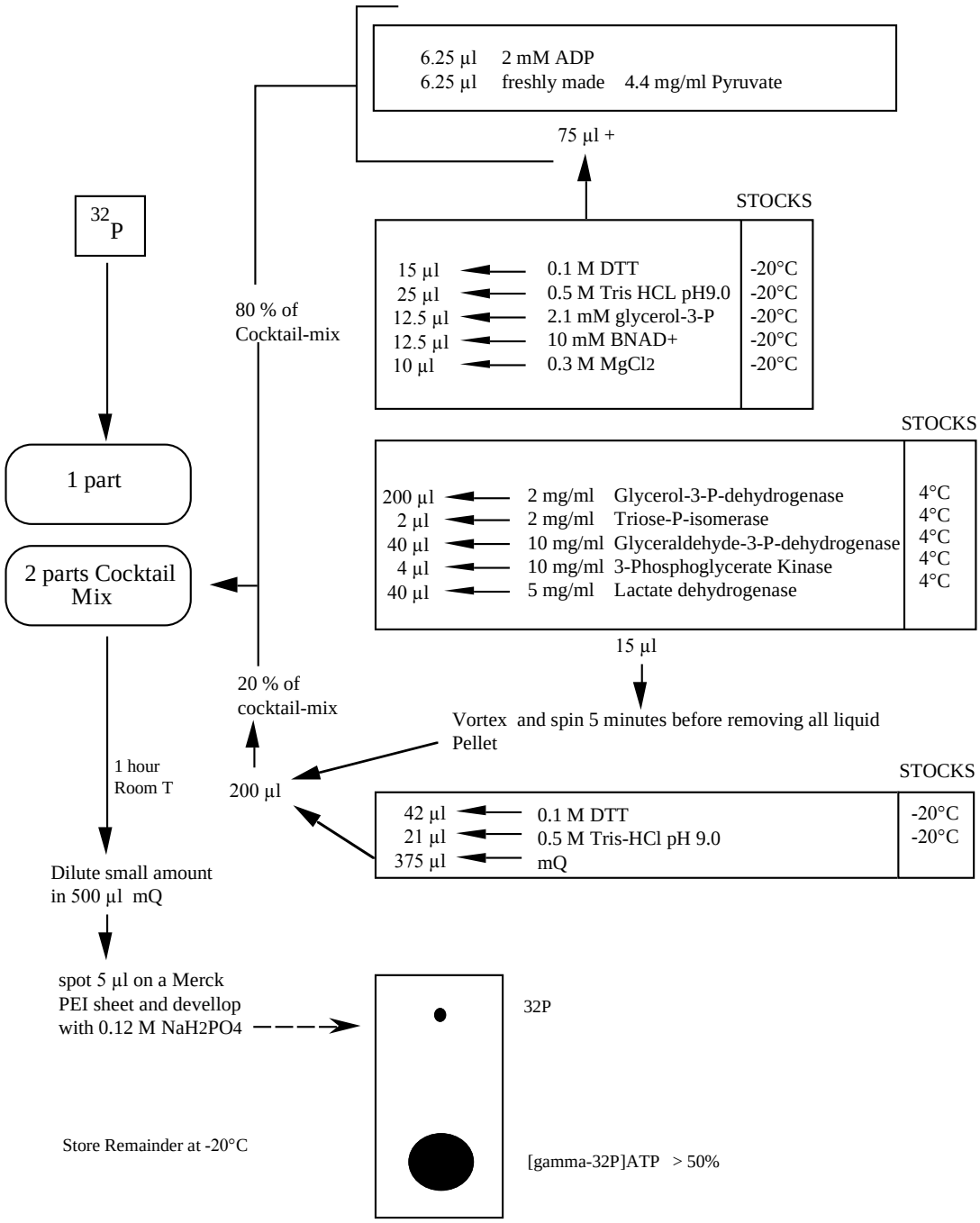
    1275 g Urea
    492 ml 98% Formic acid
    make up to 2000 ml with water

- stir and heat gently until dissolved
- titrate to pH 3.5 with solid LiOH (caution: caustic, wear eye and hand protection)
- during titration keep temperature at about 22°C, i.e. add 5 g at a time, put in ice-water if needed.
- if you go too far with pH, add one drop of formic acid, then readjust pH at 22°C
- adjust final volume to 2.5 litre with water

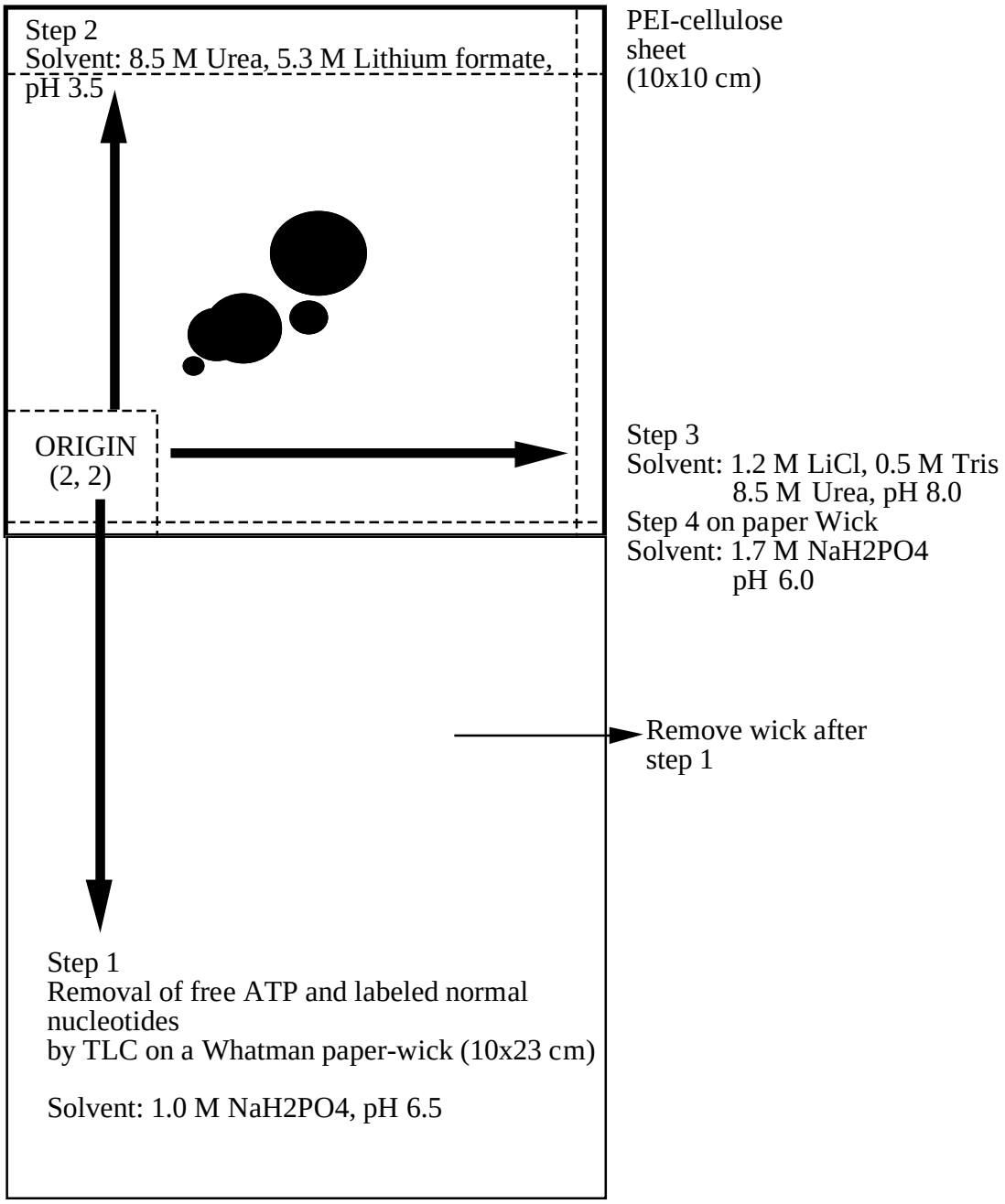
D3:
1.2 M LiCl
0.5 M Tris
8.5 M Urea
pH 8.0
- use 125 g LiCl, 150 g Tris base, and 1275 g urea
- make up to about 2000 ml with water
- titrate to pH 8.0 with concentrated HCl, letting solution cool before completing titration
- adjust volume to 2.5 litre with water

D4:
1.7 M NaH_2PO_4
pH 6.0
- use 586 g $NaH_2PO_4.H_2O$
- dissolve in about 2000 ml water
- adjust with 10 M NaOH to pH 6.0, letting solution cool before complete titration
- adjust to 2.5 litre with water

^{32}P-ATP synthesis



(i.) Chromatography of DNA-adducts



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APPENDIX 4: Phase 1 Ethics Approval

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

March 5, 2010

This Ethics Application was subject to:

☐ Full Board Review
Meeting Date:
☒ Expedited Review

Dr. Will D. King
Department of Community Health and Epidemiology
Queen's University

Dear Dr. King,

Study Title: **Dietary factors and cancer risk – a pilot study of biomarkers of exposure and early biologic effect**
Co-Investigators: **Dr. T. Massey, Ms. V. Ho**

I am writing to acknowledge receipt of your recent ethics submission. We have examined the protocol, information pamphlet, questionnaire, poster and consent form for your project (as stated above) and consider it to be ethically acceptable. This approval is valid for one year from the date of the Chair's signature below. This approval will be reported to the Research Ethics Board. Please attend carefully to the following list of ethics requirements you must fulfill over the course of your study:

- **Reporting of Amendments**: If there are any changes to your study (e.g. consent, protocol, study procedures, etc.), you must submit an amendment to the Research Ethics Board for approval. (see http://www.queensu.ca/vpr/reb.htm).

- **Reporting of Serious Adverse Events**: Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other serious adverse events must be reported within 15 days after becoming aware of the information.

- **Reporting of Complaints**: Any complaints made by participants or persons acting on behalf of participants must be reported to the Research Ethics Board within 7 days of becoming aware of the complaint. Note: All documents supplied to participants must have the contact information for the Research Ethics Board.

- **Annual Renewal**: Prior to the expiration of your approval (which is one year from the date of the Chair's signature below), you will be reminded to submit your renewal form along with any new changes or amendments you wish to make to your study. If there have been no major changes to your protocol, your approval may be renewed for another year.

Yours sincerely,

Albert Clark
Chair, Research Ethics Board

March 8, 2010
Date

ORIGINAL TO INVESTIGATOR - COPY TO DEPARTMENT HEAD- COPY TO HOSPITAL(S) /P&T (If appropriate) - FILE COPY

Study Code: EPID-305-10

- **Investigators please note that if your trial is registered by the sponsor, you must take responsibility to ensure that the registration information is accurate and complete**

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

The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards as defined by the Tri-Council Policy Statement; Part C Division 5 of the Food and Drug Regulations, OHRP, and U.S DHHS Code of Federal Regulations Title 45, Part 46 and carries out its functions in a manner consistent with Good Clinical Practices.

Federalwide Assurance Number : #FWA00004184
#IRB00001173

Current 2009 membership of the Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board

Dr. A.F. Clark	Emeritus Professor, Department of Biochemistry, Faculty of Health Sciences, Queen's University (Chair)
Dr. H. Abdollah	Professor, Department of Medicine, Queen's University
Rev. T. Deline	Community Member
Dr. M. Evans	Community Member
Dr. S. Irving	Psychologist, Providence Care, St. Mary's of the Lake Hospital Site
Prof. L. Keeping-Burke	Assistant Professor, School of Nursing, Queen's University
Mrs. J. Kotecha	Research & Programs Manager, Centre for Studies in Primary Care, Department of Family Medicine, Queen's University
Dr. J. Low	Emeritus Professor, Department of Obstetrics and Gynaecology, Queen's University and Kingston General Hospital
Dr. W. Racz	Emeritus Professor, Department of Pharmacology & Toxicology, Queen's
Dr. B. Simchison	Assistant Professor, Department of Anesthesiology, Queen's University
Dr. A.N. Singh	WHO Professor in Psychosomatic Medicine and Psychopharmacology Professor of Psychiatry and Pharmacology Chair and Head, Division of Psychopharmacology, Queen's University Director & Chief of Psychiatry, Academic Unit, Quinte Health Care, Belleville General Hospital
Dr. E. Tsai	Associate Professor, Department of Paediatrics and Office of Bioethics, Queen's University
Rev. J. Warren	Community Member
Ms. K. Weisbaum	LL.B. and Adjunct Instructor, Department of Family Medicine (Bioethics)
Dr. S. Wood	Director, Office of Research Services (Ex-Officio)

APPENDIX 5: Phase 2 Study Questionnaire

KGH KINGSTON GENERAL HOSPITAL

HOTEL DIEU HOSPITAL, KINGSTON

Queen's UNIVERSITY

Study ID# _____

CR# _____

A study of blood and tissue markers of colon health

This questionnaire is part of a research study being conducted to help us learn more about risk factors and markers that might be associated with colon (large bowel) health.

You do not have to answer any questions that make you feel uncomfortable. The answers that you provide will remain strictly confidential.

You will be identified by a study ID number and files containing your answers will contain no identifying information. Your honesty is important for the success of this research. Answer each question as best you can – estimate if you aren't sure.

Thank you very much for your participation in this study.

Please check the boxes where choices are given and write in your answers where space is provided.

1. What gender are you? ☐ Female ☐ Male
2. What is your current age? _____ years
3. How would you best describe **your grandparents'** race, ethnicity or colour? Please specify as many as applicable: ☐ **All White**

Race, ethnicity or colour	**Your Maternal Grandmother (Mother's mother)**	**Your Maternal Grandfather (Mother's father)**	**Your Paternal Grandmother (Father's mother)**	**Your Paternal Grandfather (Father's father)**
White	☐	☐	☐	☐
Black	☐	☐	☐	☐
First Nations, Natives or Aboriginal peoples of North America	☐	☐	☐	☐
Chinese	☐	☐	☐	☐
Japanese	☐	☐	☐	☐
Filipino	☐	☐	☐	☐
Korean	☐	☐	☐	☐
South Asian (e.g. East Indian, Pakistani, Punjabi, Sri Lankan)	☐	☐	☐	☐
South East Asian (e.g. Cambodian, Indonesian, Laotian, Vietnamese)	☐	☐	☐	☐
Arab/West Asian (e.g. Armenian, Egyptian, Iranian, Lebanese, Moroccan)	☐	☐	☐	☐
Latin American	☐	☐	☐	☐
Other (specify)	☐	☐	☐	☐

LIFESTYLE QUESTIONS

4. Please indicate the **number of cups** or glasses of the following beverages (1cup=250 ml or 8 oz) that you drank **on average over the past year**. Please put a check mark in the box in the appropriate column. ☑ (Choose one column for each type of beverage.)

Type of Beverage	**None**	**Less than 1 per week**	**1-6 per week**	**1 per day**	**2 per day**	**3 per day**	**4 or more per day**
Coffee containing caffeine	☐	☐	☐	☐	☐	☐	☐
Decaffeinated coffee	☐	☐	☐	☐	☐	☐	☐
Tea containing caffeine	☐	☐	☐	☐	☐	☐	☐
Decaffeinated tea	☐	☐	☐	☐	☐	☐	☐
Soft drinks containing caffeine	☐	☐	☐	☐	☐	☐	☐

5. During the past year have you had **at least one** drink of beer, wine, liquor **or** coolers?

1 drink = 12 oz beer or cooler (1 bottle), 5 oz wine (1 glass) or 1.5 oz hard liquor (spirits)

☐ No *(Go to question 6)*

☐ Yes ➡ Indicate in the chart below how many drinks you consumed, **on average each week over the past year.** Please put a check mark in the box in the appropriate column.☑ (Choose one column for each type of beverage.)

Type of Beverage	**None**	**Less than 1 per week**	**1-7 per week**	**8-14 per week**	**15-21 per week**	**More than 21 per week**
Beer (12 oz)	☐	☐	☐	☐	☐	☐
Wine (5 oz)	☐	☐	☐	☐	☐	☐
Liquor (1.5 oz)	☐	☐	☐	☐	☐	☐
Coolers, breezers (12 oz)	☐	☐	☐	☐	☐	☐

6. During the **past year** have you smoked at least 1 cigarette a day?

☐ No *(Go to question 7)*

☐ Yes ➡ On average, how many cigarettes did you usually smoke in one day?
____ cigarettes per day

7. Have you **ever** smoked at least 1 cigarette a day for 6 months or more?

☐ No *(Go to question 8)*

☐ Yes ➡ a) How old were you when you began to smoke? ____ years old

b) When you smoked the most, how many cigarettes did you usually smoke in one day?
____ cigarettes per day

c) Are you still smoking?

☐ No ➡ How old were you when you stopped smoking? ____ years old

☐ Yes

8. "Second-hand" smoke exposure occurs when you are close enough to a person smoking to smell the tobacco smoke. **During the past year**, how many hours **per week** were you exposed to second hand smoke?

☐ Never or < 1 hour per week

☐ 1-4 hours

☐ 5-9 hours

☐ 10-14 hours

☐ 15 or more hours

PHYSICAL ACTIVITY QUESTIONS

We are interested in finding out about the kinds of physical activity that you have done in a **usual week over the past year.** Think about occupational, household, volunteer, sport, recreational and leisure activities.

Think about all the **vigorous or strenuous** activities that you did in an average or usual week in the past year. **Vigorous** physical activities refer to activities that take hard physical effort, increase your heart rate substantially, cause heavy sweating and make you breathe much harder than normal. Think *only* about those physical activities that you did for **at least 10 minutes at a time**.

9. During a usual week in the past year, on how many **days** did you do vigorous physical activities such as major house cleaning, gardening or home repairs, strenuous occupation-related activities, vigorous child care, or sports such as fast cycling, hockey, basketball, soccer, jogging, aerobics or racquet sports for at least 10 minutes at a time?

   _____ days per week

   ☐ No vigorous physical activities ➡ Skip to question 11

10. **On average,** how much time did you usually spend doing vigorous physical activities on **one of those days**?

    _____ hours per day **OR** _____ minutes per day

Think about all the activities which take **moderate physical effort** that you did in an average or usual week in the past year. **Moderate** activities refer to activities that are not exhausting but increase your heart rate slightly, make you breathe somewhat harder than normal and may cause some light perspiration. Think *only* about those physical activities that you did for **at least 10 minutes at a time.**

11. During a usual week in the past year, on how many **days** did you do moderate physical activities such as light effort house cleaning, gardening, home repairs or occupation-related activities, or sports such as power walking, hiking, bicycling at a regular pace, golf, curling, social dancing, softball or volleyball for at least 10 minutes at a time?

    _____ days per week

    ☐ No moderate physical activities ➡ Skip to question 13

12. **On average,** how much time did you usually spend doing moderate physical activities on **one of those days**?

    _____ hours per day **OR** _____ minutes per day

Think about the time you spent **walking at a casual or normal pace** in a usual week in the past year. This includes walking to and/or from work or other activities, and any other walking that you might have done as part of your job or for recreation, sport, exercise, or leisure.

13. During a usual week in the past year, on how many days did you walk at a casual or normal pace for **at least 10 minutes at a time**? _____ days per week

    ☐ No walking ➡ Skip to question 15

14. **On average,** how much time did you usually spend walking on **one of those days**?

    _____ hours per day **OR** _____ minutes per day

PAST MEDICAL HISTORY

15. Were you ever told by a family doctor/general practitioner or specialist that you had any of the following conditions or symptoms?

Medical Condition	Yes	No
Angina (Coronary Artery Disease)	☐	☐
High blood pressure (Hypertension)	☐	☐
Heart Attack (Myocardial Infarction)	☐	☐
Irritable bowel syndrome	☐	☐
Type 1 Diabetes	☐	☐
Type 2 Diabetes	☐	☐
Diabetes: Type unknown	☐	☐

FAMILY HISTORY OF COLORECTAL CANCER

16. We would like to know if either of your parents or any of your full siblings (brothers or sisters) or biological children has ever had **colorectal cancer** (cancer of the large bowel). Please record the earliest (youngest) age at diagnosis if more than one sibling or child has had colorectal cancer. *(Full siblings are brothers and sisters with **both** the same mother and father as you.)*

Relative	Have they ever been diagnosed with colorectal cancer?			Approximate age (in years) of relative at time of their diagnosis
Mother	☐ Yes	☐ No	☐ Don't know	_____
Father	☐ Yes	☐ No	☐ Don't know	_____
Sibling	☐ Yes	☐ No	☐ Don't know	_____
Child	☐ Yes	☐ No	☐ Don't know	_____

MEDICATION USE

The next 3 questions ask about your use of specific medications over the past 10 years.

17. **Acetaminophen-containing medications:** Examples of non-prescription medications that include acetaminophen include tylenol, panadol and exdol as well as other drug store brand names. **In the last 10 years**, have you ever taken any acetaminophen-containing medications **at least 4 times per week on a regular basis (that is, every week) for 3 or more consecutive months?**

    ☐ No (*Go to question 18*)

    ☐ Yes → a) Thinking back to the **past year only**: In total, how many months did you take any of these acetaminophen-containing medications **at least 4 times per week**? _____ months

    b) Thinking back over the **past 10 years**: In total, how long did you take any of these acetaminophen-containing medications **at least 4 times per week**?

    _____ months **or** _____ years

18. **Non-prescription non-steroidal anti-inflammatory drugs (NSAIDs):** Examples of non-steroidal anti-inflammatory drugs (commonly known as NSAIDs for short) that **do not require a prescription** include Aspirin (ASA), Anacin, Bufferin, Entrophen, Advil, Motrin and Ibuprofen. **In the last 10 years**, have you ever taken any over-the-counter, **non-prescription NSAIDs at least 4 times per week on a regular basis (that is, every week) for 3 or more consecutive months?**

☐ No (*Go to question 19*)

☐ Yes → a) Thinking back to the **past year only**: In total, how many months did you take any of these **non-prescription** NSAID medications **at least 4 times per week**? _____ months

b) Thinking back over the **past 10 years**: In total, how long did you take any of these non-prescription NSAID medications **at least 4 times per week**?

_____ months **or** _____ years

19. **Prescription non-steroidal anti-inflammatory drugs (NSAIDs):** Examples of NSAIDs that **require a prescription** include Naprosyn, Celebrex and Voltaren. **In the last 10 years**, have you ever taken any **prescription** NSAIDs **at least 4 times per week on a regular basis (that is, every week) for 3 or more consecutive months?**

☐ No (*Go to question 20*)

☐ Yes → a) Thinking back to the **past year only**: In total, how many months did you take any of these **prescription** NSAID medications **at least 4 times per week**? _____ months

b) Thinking back over the **past 10 years**: In total, how long did you take any of these prescription NSAID medications **at least 4 times per week**?

_____ months **or** _____ years

VITAMIN AND OTHER SUPPLEMENT USE

20. **Over the last 12 months**, have you taken any of the following types of supplements or preparations on a regular basis (that is, **more than once per week for at least 6 months during the past 12 months)?** (Check off all that apply. ☑)

Supplement or preparation	YES	NO
Fiber supplements such as Metamucil, Fiber-sure, Benefiber or other fiber supplements	☐	☐
Multi-vitamins (One-a-day, Spectrum, Centrum etc.)	☐	☐
Other supplements that are **not** part of a multi-vitamin ☐ No *(Go to question 21)* ☐ Yes: Please check off all that apply from the list below.		
Vitamin A	☐	☐
Vitamin C	☐	☐
Vitamin D	☐	☐
Vitamin E	☐	☐
Zinc	☐	☐
Beta carotene	☐	☐
Calcium or calcium-containing antacid	☐	☐
Magnesium	☐	☐
Vitamin B6	☐	☐
Vitamin B12	☐	☐
Vitamin B-complex	☐	☐
Folic acid	☐	☐
Selenium	☐	☐
Iron (Palifer, Eurofer etc.)	☐	☐
Niacin	☐	☐
Other: specify_____	☐	☐

DIETARY ASSESSMENT

21. **<u>Over the last 12 months</u>**, **on average**, *how often* did you eat or drink the following dairy products. Please put a check mark in the box in the appropriate column. ☑ (Choose one column for each type of food or beverage.)

Dairy Products	***On average* how often?**						
	Never	**Less than 1 per week**	**1 per week**	**2-3 per week**	**4-6 per week**	**Once per day**	**More than once per day**
Milk (white or chocolate)							
Skim	☐	☐	☐	☐	☐	☐	☐
1% or 2%	☐	☐	☐	☐	☐	☐	☐
Whole/Homogenized	☐	☐	☐	☐	☐	☐	☐
Soy	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐
Cream (in coffee, whipped or sour cream)	☐	☐	☐	☐	☐	☐	☐
Yogurt (not frozen yogurt)	☐	☐	☐	☐	☐	☐	☐
Frozen yogurt, sherbet or low-fat ice cream	☐	☐	☐	☐	☐	☐	☐
Regular ice cream	☐	☐	☐	☐	☐	☐	☐
Cottage or ricotta cheese	☐	☐	☐	☐	☐	☐	☐
Cream cheese	☐	☐	☐	☐	☐	☐	☐
Processed cheese	☐	☐	☐	☐	☐	☐	☐
Hard cheese: low fat, light or fat-free	☐	☐	☐	☐	☐	☐	☐
Hard cheese: regular	☐	☐	☐	☐	☐	☐	☐

22. **<u>Over the last 12 months</u>**, **on average**, *how often* did you drink or eat the following fruits? Please include fresh, canned or frozen fruit. Please put a check mark in the box in the appropriate column. ☑ (Choose one column for each type of food or beverage).

Fruits (including fresh, frozen or canned)	***On average* how often?**						
	Never	**Less than 1 per week**	**1 per week**	**2-3 per week**	**4-6 per week**	**Once per day**	**More than once per day**
Orange juice	☐	☐	☐	☐	☐	☐	☐
Apples, applesauce	☐	☐	☐	☐	☐	☐	☐
Bananas	☐	☐	☐	☐	☐	☐	☐
Cantaloupe, honeydew melon or watermelon	☐	☐	☐	☐	☐	☐	☐
Oranges, tangerines, tangelos, clementines	☐	☐	☐	☐	☐	☐	☐
Grapefruit	☐	☐	☐	☐	☐	☐	☐
Strawberries, blueberries, raspberries or other berries	☐	☐	☐	☐	☐	☐	☐
Grapes	☐	☐	☐	☐	☐	☐	☐
Peaches or plums	☐	☐	☐	☐	☐	☐	☐
Dried fruits (such as plums or raisins)	☐	☐	☐	☐	☐	☐	☐

23. **<u>Over the last 12 months</u>**, **on average**, *how often* did you consume each vegetable item? Please include fresh, canned or frozen vegetables. Please put a check mark in the box in the appropriate column. ☑ (Choose one column for each type of food or beverage.)

Vegetables (including fresh, frozen or canned)	***On average* how often?**						
	Never	**Less than 1 per week**	**1 per week**	**2-3 per week**	**4-6 per week**	**Once per day**	**More than once per day**
100% Tomato or vegetable juice	☐	☐	☐	☐	☐	☐	☐
Tomatoes, tomato sauce, tomato paste etc	☐	☐	☐	☐	☐	☐	☐
Broccoli	☐	☐	☐	☐	☐	☐	☐
Asparagus	☐	☐	☐	☐	☐	☐	☐
Cauliflower	☐	☐	☐	☐	☐	☐	☐
Corn	☐	☐	☐	☐	☐	☐	☐
Peas	☐	☐	☐	☐	☐	☐	☐
Green beans	☐	☐	☐	☐	☐	☐	☐
Cooked greens such as spinach, collard, chard, kale	☐	☐	☐	☐	☐	☐	☐
Lettuce or other salad greens including spinach	☐	☐	☐	☐	☐	☐	☐
Carrots	☐	☐	☐	☐	☐	☐	☐
Celery, cucumber	☐	☐	☐	☐	☐	☐	☐
Potatoes (not fried)	☐	☐	☐	☐	☐	☐	☐
Sweet potatoes, yams, zucchini, onions, turnip	☐	☐	☐	☐	☐	☐	☐

24. **<u>Over the last 12 months</u>**, **on average**, *how often* did you eat each of the following meats or alternative items specified? Please put a check mark in the box in the appropriate column. ☑ (Choose one column for each type of food.)

Meat and alternatives	***On average* how often?**						
	Never	**Less than 1 per week**	**1 per week**	**2-3 per week**	**4-6 per week**	**Once per day**	**More than once per day**
Beef liver	☐	☐	☐	☐	☐	☐	☐
Lunch (sandwich) meat or cold cuts such as bologna, salami, ham	☐	☐	☐	☐	☐	☐	☐
Hot dogs or sausage	☐	☐	☐	☐	☐	☐	☐
Fish (canned)	☐	☐	☐	☐	☐	☐	☐
Eggs	☐	☐	☐	☐	☐	☐	☐
Cooked dried beans including baked beans, kidney, lima bea lentils, soybeans etc	☐	☐	☐	☐	☐	☐	☐
Tofu	☐	☐	☐	☐	☐	☐	☐
Nuts or seeds	☐	☐	☐	☐	☐	☐	☐

25. **<u>Over the last 12 months</u>**, **on average**, *how often* did you eat each of the following grain products? Please put a check mark in the box in the appropriate column. ☑ (Choose one column for each type of food.)

Grain products (breads and cereals)	***On average* how often?**						
	Never	**Less than 1 per week**	**1 per week**	**2-3 per week**	**4-6 per week**	**Once per day**	**More than once per day**
Bran breakfast cereals (not cooked)	☐	☐	☐	☐	☐	☐	☐
Other uncooked breakfast cereals	☐	☐	☐	☐	☐	☐	☐
Cooked oatmeal or bran cereal	☐	☐	☐	☐	☐	☐	☐
Other cooked cereal	☐	☐	☐	☐	☐	☐	☐
Rolls, bread or bagels: whole grain, whole wheat, multigrain or oatmeal	☐	☐	☐	☐	☐	☐	☐
Rolls, bread or bagels: rye or pumpernickel	☐	☐	☐	☐	☐	☐	☐
Other bread, rolls or bagels (white, Italian, French, egg or raisin)	☐	☐	☐	☐	☐	☐	☐
Brown rice	☐	☐	☐	☐	☐	☐	☐
White rice	☐	☐	☐	☐	☐	☐	☐
Whole wheat or multigrain pasta	☐	☐	☐	☐	☐	☐	☐
White pasta	☐	☐	☐	☐	☐	☐	☐
Other cooked grains (couscous, quinoa etc)	☐	☐	☐	☐	☐	☐	☐

26. Thinking about the **past summer**, indicate **how often** you ate the following types of cooked meat and fish, **AND** the typical **level of doneness**.

	How often?							Level of doneness		
	Never	**Less than 1 per month**	**1-3 per month**	**1 per week**	**2-3 per week**	**4-6 per week**	**7+ per week**	**Rare**	**Medium**	**Well-done**
Beef Steak										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hamburger										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Never	**Less than 1 per month**	**1-3 per month**	**1 per week**	**2-3 per week**	**4-6 per week**	**7+ per week**	**Just until done**	**Well-done**	**Very well-done**
Pork										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Chicken										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fish										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

27. Thinking about the **past winter**, indicate **how often** you ate the following types of cooked meat and fish, **AND** the typical **level of doneness**.

	How often?							Level of doneness		
	Never	**Less than 1 per month**	**1-3 per month**	**1 per week**	**2-3 per week**	**4-6 per week**	**7+ per week**	**Rare**	**Medium**	**Well-done**
Beef Steak										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hamburger										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Never	**Less than 1 per month**	**1-3 per month**	**1 per week**	**2-3 per week**	**4-6 per week**	**7+ per week**	**Just until done**	**Well-done**	**Very well-done**
Pork										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Chicken										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fish										
Pan-fried	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Grilled or Barbequed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Oven-broiled	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

MENSTRUAL AND REPRODUCTIVE QUESTIONS

28. How old were you when you had your first menstrual period? _____ years of age

    ☐ Have never menstruated (G*o to question 30*)

29. Are you still menstruating?

    ☐ Yes (*Go to question 31*)

    ☐ No → a) How many months **or** years has it been since your last menstrual period?
    _____ month(s) **or** _____ year(s)

    b) How old were you when you had your last menstrual period?
    _____ years of age

    c) How did your menstrual periods stop?

    ☐ Naturally (through onset of menopause)

    ☐ As a result of a hysterectomy

    ☐ As a result of radiation or chemotherapy

    ☐ Other – please specify: _____

30. Have you ever had a hysterectomy (that is, an operation to remove the womb/uterus)?

    ☐ No

    ☐ Yes → at what age? _____ years

31. Have you ever had an oophorectomy (that is, an operation to remove one or both of your ovaries which is sometimes done at the same time as removal of the womb/uterus)?

    ☐ No (*Go to question 32*)

    ☐ Yes → ☐ one ovary removed: → at what age? _____ years

    ☐ second ovary removed: → at what age? _____ years

    or

    ☐ both ovaries removed at same time: → at what age? _____ years

    ☐ Don't know

32. Have you ever taken prescribed birth control medication for birth control or any other medical reason **for 6 or more consecutive months**?

☐ No (*Go to question 33*)

☐ Yes → a) How old were you when you **first** took birth control medication for **6 months or more**? _____ years

b) Are you still taking birth control medication?

☐ No → At what age did you stop for the **last** time? _____ years

☐ Yes

c) In total, how long did you take/have you taken birth control medication, **excluding** the times when you took a break from taking the medication? (*Please give the total time EITHER in months OR in years*.) _____ months **or** _____ years

33. Have you ever taken any type of female replacement hormones (presently known as hormone therapy or HT and previously called hormone replacement therapy or HRT) **for 6 or more consecutive months**? *Please consider all methods of use, such as oral (pill), skin patch, injection or vaginal cream routes of administration.*

☐ No (*Go to question 34*)

☐ Yes → a) How old were you when you first started taking these hormones? _____ years

b) Thinking back to the past year **only**: In total, during this 1 year period, for how many months did you take any type of hormone therapy? _____ months

c) Thinking back over the past 10 years: In total, counting all the types of hormone therapy used, how many months or years did you take/have you taken these medications?
_____ months **or** _____ years

34. Have you ever been pregnant? (include live births, still births, miscarriages and abortions)

☐ No

☐ Yes → a) How many times have you been pregnant? (*Include all live births, stillbirths, miscarriages, abortions, ectopic (tubal) pregnancies. Twins or triplets count as one pregnancy)* _____ number of pregnancies

b) How old were you at the beginning of the **first** pregnancy that lasted **more than 6 months**? _____ years

c) How many of your pregnancies were live births? _____ number of live births

APPENDIX 6: Phase 2 Ethics Approval

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

Queen's UNIVERSITY

September 1, 2011

This Ethics Application was subject to:

☐ Full Board Review
Meeting Date:
☒ Expedited Review

Dr. Will King
Department of Community Health and Epidemiology
Queen's University

Dear Dr. King,

Study Title: **Meat-derived carcinogens and colorectal adenoma risk**
Co-Investigators: **Dr. T. Massey, Dr. J. Ashbury, Dr. S. Vanner, Ms. V. Ho, Ms. S. Peacock**

I am writing to acknowledge receipt of your recent ethics submission. We have examined the protocol for your project (as stated above) and consider it to be ethically acceptable. This approval is valid for one year from the date of the Chair's signature below. This approval will be reported to the Research Ethics Board. Please attend carefully to the following list of ethics requirements you must fulfill over the course of your study:

- **Reporting of Amendments:** If there are any changes to your study (e.g. consent, protocol, study procedures, etc.), you must submit an amendment to the Research Ethics Board for approval. (see http://www.queensu.ca/vpr/reb.htm).

- **Reporting of Serious Adverse Events:** Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other serious adverse events must be reported within 15 days after becoming aware of the information.

- **Reporting of Complaints:** Any complaints made by participants or persons acting on behalf of participants must be reported to the Research Ethics Board within 7 days of becoming aware of the complaint. Note: All documents supplied to participants must have the contact information for the Research Ethics Board.

- **Annual Renewal:** Prior to the expiration of your approval (which is one year from the date of the Chair's signature below), you will be reminded to submit your renewal form along with any new changes or amendments you wish to make to your study. If there have been no major changes to your protocol, your approval may be renewed for another year.

Yours sincerely,

Albert Clark
Chair, Research Ethics Board

Sept 1, 2011
Date

ORIGINAL TO INVESTIGATOR - COPY TO DEPARTMENT HEAD- COPY TO HOSPITAL – BINDER COPY - FILE COPY

Study Code: EPID-354-11

- **Investigators please note that if your trial is registered by the sponsor, you must take responsibility to ensure that the registration information is accurate and complete**

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

The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards as defined by the Tri-Council Policy Statement; Part C Division 5 of the Food and Drug Regulations, OHRP, and U.S DHHS Code of Federal Regulations Title 45, Part 46 and carries out its functions in a manner consistent with Good Clinical Practices.

Federalwide Assurance Number : #FWA00004184
#IRB00001173

Current 2011 membership of the Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board

Dr. A.F. Clark	Emeritus Professor, Department of Biochemistry, Faculty of Health Sciences, Queen's University (Chair)
Dr. H. Abdollah	Professor, Department of Medicine, Queen's University
Dr. R. Brison	Professor, Department of Emergency Medicine, Queen's University
Dr. M. Evans	Community Member
Dr. S. Horgan	Manager, Program Evaluation & Health Services Development, Geriatric Psychiatry Service, Providence Care, Mental Health Services Assistant Professor, Department of Psychiatry
Dr. B. Kisilevsky	Professor, School of Nursing, Departments of Psychology and Obstetrics & Gynaecology, Queen's University
Ms. D. Morales	Community Member
Dr. W. Racz	Emeritus Professor, Department of Pharmacology & Toxicology, Queen's
Ms. S. Rohland	Privacy Officer, ICES-Queen's Health Services Research Facility Research Associate, Division of Cancer Care and Epidemiology, Queen's Cancer Research Institute
Dr. B. Simchison	Assistant Professor, Department of Anesthesiology, Queen's University
Dr. A.N. Singh	WHO Professor in Psychosomatic Medicine and Psychopharmacology Professor of Psychiatry and Pharmacology Chair and Head, Division of Psychopharmacology, Queen's University Director & Chief of Psychiatry, Academic Unit, Quinte Health Care, Belleville General Hospital
Dr. E. Tsai	Associate Professor, Department of Paediatrics and Office of Bioethics, Queen's University
Rev. J. Warren	Community Member

APPENDIX 7: Phase 2 Additional Results

Table 1. Frequency of categorical covariates among dichotomous representation of PhIP

Covariate	Categories	Categories 1 and 2 PhIP≤232.61 ng/day (%)	Categories 3 and 4 PhIP>232.61 ng/day (%)	p[1]
Sex	Female	110 (57.0)	88 (59.1)	0.70
	Male	83 (43.0)	61 (40.9)	
Age	39-50	28 (14.5)	33 (22.2)	0.14
	50-60	119 (61.7)	79 (53.0)	
	60+	46 (23.8)	37 (24.8)	
Ethnicity	White	180 (93.8)	140 (94.0)	0.94
	Other	12 (6.2)	9 (6.0)	
Smoking Status	Never	112 (58.0)	71 (47.7)	0.11
	Past	56 (29.0)	59 (39.6)	
	Current	25 (13.0)	19 (12.7)	
Environmental Tobacco Smoke	Unexposed	146 (76.0)	112 (75.7)	0.94
	Exposed	46 (24.0)	36 (24.3)	
Family History of CRC	Yes	114 (60.1)	100 (67.1)	0.13
	No	79 (40.9)	49 (32.9)	
Previous Diagnosis of IBS	Previous Diagnosis	15 (7.8)	21 (14.1)	0.06
	No Diagnosis	178 (92.2)	128 (85.9)	
Physical Activity (MET min/week)	≤1865	47 (24.6)	42 (28.2)	0.61
	1866-3913	51 (26.7)	38 (25.5)	
	3914-6363	46 (24.1)	28 (18.8)	
	≥6364	47 (24.6)	41 (27.5)	
Caffeine Intake (mg/day)	<107.02	38 (25.5)	39 (20.3)	0.59
	107.03-207.54	33 (22.2)	47 (24.5)	
	207.55-370.71	45 (30.2)	55 (28.7)	
	≥370.72	33 (22.1)	51 (26.5)	
Alcohol Intake (g/day)	Abstainers	28 (14.5)	26 (17.5)	0.59
	Low	102 (52.9)	81 (54.3)	
	Moderate	63 (32.6)	42 (28.2)	

Dietary Energy Intake (kcal/day)	≤1017.02 1017.03-1388.49 1388.50-1781.56 ≥1781.57	56 (29.0) 52 (26.9) 43 (22.3) 42 (21.8)	36 (24.2) 35 (23.5) 41 (27.5) 37 (24.8)	0.49
Fruit and Vegetable Intake (servings per day)	≤2.9 3.0-4.1 4.2-6.0 ≥6.1	49 (25.4) 56 (29.0) 51 (26.4) 37 (19.2)	39 (26.2) 24 (16.1) 48 (32.2) 38 (25.5)	0.04
Serum Albumin (g/L)	≤40.0 40.1-42.0 42.1-44.0 ≥44.1	57 (30.2) 39 (20.6) 45 (23.8) 48 (25.4)	34 (23.1) 42 (28.6) 42 (28.6) 29 (19.7)	0.14
Serum Folate (nmol/L)	≤26.6 26.7-37.3 37.4-45.3 ≥45.4	46 (24.3) 54 (28.6) 30 (15.9) 59 (31.2)	37 (25.1) 38 (25.9) 31 (21.1) 41 (27.9)	0.62
Serum Cholesterol (mmol/L)	≤4.24 4.25-4.96 4.97-5.75 ≥5.76	57 (30.2) 42 (22.2) 48 (25.4) 42 (22.2)	32 (21.8) 32 (21.8) 36 (24.4) 47 (32.0)	0.16

[1]p-value obtained from a χ^2 test

Table 2. Frequency of categorical covariates among dichotomous representation of MeIQx

Covariate	Categories	Categories 1 and 2 MeIQx≤31.09 ng/day (%)	Categories 3 and 4 MeIQx>31.09 ng/day (%)	p[1]
Sex	Female	121 (60.5)	77 (45.8)	0.25
	Male	79 (39.5)	65 (54.2)	
Age	39-50	29 (14.5)	32 (22.5)	0.15
	50-60	119 (59.5)	79 (55.6)	
	60+	52 (26.0)	31 (21.9)	
Ethnicity	White	190 (95.0)	130 (92.2)	0.29
	Other	10 (5.0)	11 (7.8)	
Smoking Status	Never	118 (59.0)	65 (45.8)	0.05
	Past	60 (30.0)	55 (38.7)	
	Current	22 (11.0)	22 (15.5)	
Environmental Tobacco Smoke	Unexposed	160 (80.4)	98 (69.5)	0.02
	Exposed	39 (19.6)	43 (30.5)	
Family History of CRC	Yes	122 (61.0)	92 (64.8)	0.48
	No	78 (39.0)	50 (35.2)	
Previous Diagnosis of IBS	Previous Diagnosis	22 (11.0)	14 (9.9)	0.74
	No Diagnosis	178 (89.0)	128 (90.1)	
Physical Activity (MET min/week)	≤1865	55 (27.8)	34 (23.9)	0.43
	1866-3913	55 (27.8)	34 (23.9)	
	3914-6363	43 (21.7)	31 (21.8)	
	≥6364	45 (22.7)	43 (30.4)	
Caffeine Intake (mg/day)	<107.02	46 (23.1)	31 (21.8)	0.27
	107.03-207.54	52 (26.1)	28 (19.7)	
	207.55-370.71	59 (29.7)	41 (28.9)	
	≥370.72	42 (21.1)	42 (29.6)	
Alcohol Intake (g/day)	Abstainers	32 (16.0)	22 (15.5)	0.16
	Low	99 (49.5)	84 (59.1)	
	Moderate	69 (34.5)	36 (25.4)	
Dietary Energy Intake (kcal/day)	≤1017.02	55 (27.5)	37 (26.0)	0.89
	1017.03-1388.49	52 (26.0)	35 (24.7)	
	1388.50-1781.56	46 (23.0)	38 (26.8)	
	≥1781.57	47 (23.5)	32 (22.5)	

Fruit and Vegetable Intake (servings per day)	≤2.9	48 (24.0)	40 (28.2)	0.49
	3.0-4.1	48 (24.0)	32 (22.5)	
	4.2-6.0	55 (27.5)	44 (31.0)	
	≥6.1	49 (24.5)	26 (18.3)	
Serum Albumin (g/L)	≤40.0	49 (25.1)	42 (29.8)	0.65
	40.1-42.0	47 (24.1)	34 (24.1)	
	42.1-44.0	55 (28.2)	32 (22.7)	
	≥44.1	44 (22.6)	33 (23.4)	
Serum Folate (nmol/L)	≤26.6	40 (20.5)	43 (30.5)	0.22
	26.7-37.3	56 (28.7)	36 (25.5)	
	37.4-45.3	37 (19.0)	24 (17.0)	
	≥45.4	62 (31.8)	38 (27.0)	
Serum Cholesterol (mmol/L)	≤4.24	51 (26.2)	38 (27.9)	0.72
	4.25-4.96	47 (24.1)	27 (19.3)	
	4.97-5.75	46 (23.6)	38 (27.9)	
	≥5.76	51 (26.1)	38 (27.9)	

[1]p-value obtained from a χ^2 test

Table 3. Frequency of categorical covariates among dichotomous representation of DiMeIQx

Covariate	Categories	Categories 1 and 2 DiMeIQx≤4.12 ng/day (%)	Categories 3 and 4 DiMeIQx>4.12 ng/day (%)	p[1]
Sex	Female	122 (60.4)	76 (54.3)	0.26
	Male	80 (39.6)	64 (45.7)	
Age	39-50	30 (14.9)	31 (22.1)	0.22
	50-60	121 (59.9)	77 (55.0)	
	60+	51 (25.2)	32 (22.9)	
Ethnicity	White	194 (96.0)	126 (90.7)	0.04
	Other	8 (4.0)	13 (9.3)	
Smoking Status	Never	118 (58.4)	65 (46.4)	0.04
	Past	5 (28.2)	58 (41.4)	
	Current	27 (13.4)	17 (12.2)	
Environmental Tobacco Smoke	Unexposed	155 (77.1)	103 (74.1)	0.52
	Exposed	46 (25.9)	36 (22.9)	
Family History of CRC	Yes	122 (60.4)	92 (65.7)	0.32
	No	80 (39.6)	48 (34.3)	
Previous Diagnosis of IBS	Previous Diagnosis	20 (9.9)	16 (11.4)	0.65
	No Diagnosis	182 (90.1)	124 (88.6)	
Physical Activity (MET min/week)	≤1865	53 (26.5)	36 (25.7)	0.73
	1866-3913	56 (28.0)	33 (23.6)	
	3914-6363	43 (21.5)	31 (22.1)	
	≥6364	48 (24.0)	40 (28.6)	
Caffeine Intake (mg/day)	<107.02	46 (22.9)	31 (22.1)	0.84
	107.03-207.54	48 (23.9)	32 (22.9)	
	207.55-370.71	61 (30.3)	39 (27.9)	
	≥370.72	46 (22.9)	38 (27.1)	
Alcohol Intake (g/day)	Abstainers	29 (14.4)	25 (17.9)	0.23
	Low	104 (51.5)	79 (54.4)	
	Moderate	69 (34.1)	36 (25.7)	
Dietary Energy Intake (kcal/day)	≤1017.02	53 (26.2)	39 (27.9)	0.93
	1017.03-1388.49	50 (24.8)	37 (26.4)	
	1388.50-1781.56	52 (25.7)	32 (22.9)	
	≥1781.57	47 (23.3)	32 (22.8)	

Fruit and Vegetable Intake (servings per day)	≤2.9	48 (23.8)	40 (28.6)	0.55
	3.0-4.1	51 (25.2)	29 (20.7)	
	4.2-6.0	56 (27.7)	43 (30.7)	
	≥6.1	47 (23.3)	28 (20.0)	
Serum Albumin (g/L)	≤40.0	55 (27.9)	36 (25.9)	0.78
	40.1-42.0	46 (23.3)	35 (25.2)	
	42.1-44.0	48 (24.4)	39 (28.0)	
	≥44.1	48 (24.4)	29 (24.9)	
Serum Folate (nmol/L)	≤26.6	48 (24.4)	35 (25.2)	0.48
	26.7-37.3	57 (28.9)	35 (25.2)	
	37.4-45.3	39 (19.8)	22 (15.8)	
	≥45.4	53 (26.9)	47 (33.8)	
Serum Cholesterol (mmol/L)	≤4.24	57 (28.9)	32 (23.1)	0.28
	4.25-4.96	47 (23.9)	27 (19.4)	
	4.97-5.75	47 (23.9)	37 (26.6)	
	≥5.76	46 (23.3)	43 (30.9)	

[1]p-value obtained from a χ^2 test

Table 4. Frequency of categorical covariates among dichotomous representation of meat mutagenicity exposure

Covariate	Categories	Categories 1 and 2 Meat Mutagenicity ≤7179.67 ng/day (%)	Categories 3 and 4 Meat Mutagenicity >7179.67 ng/day (%)	p[1]
Sex	Female	117 (61.3)	81 (53.6)	0.16
	Male	74 (38.7)	70 (46.4)	
Age	39-50	25 (13.1)	36 (23.8)	0.04
	50-60	118 (61.8)	80 (53.0)	
	60+	48 (25.1)	35 (23.2)	
Ethnicity	White	180 (94.7)	140 (92.7)	0.44
	Other	10 (5.3)	11 (7.3)	
Smoking Status	Never	109 (57.1)	74 (49.0)	0.24
	Past	57 (29.8)	58 (38.4)	
	Current	25 (13.1)	19 (12.6)	
Environmental Tobacco Smoke	Unexposed	148 (77.9)	110 (73.3)	0.33
	Exposed	42 (22.1)	40 (26.7)	
Family History of CRC	Yes	116 (60.7)	98 (64.9)	0.43
	No	75 (39.3)	53 (35.1)	
Previous Diagnosis of IBS	Previous Diagnosis	17 (8.9)	19 (12.6)	0.27
	No Diagnosis	174 (91.1)	132 (87.4)	
Physical Activity (MET min/week)	≤1865	54 (28.6)	35 (23.2)	0.38
	1866-3913	48 (25.4)	41 (27.1)	
	3914-6363	44 (23.3)	30 (19.9)	
	≥6364	43 (22.7)	45 (29.8)	
Caffeine Intake (mg/day)	<107.02	39 (20.5)	38 (25.2)	0.38
	107.03-207.54	51 (26.8)	29 (19.2)	
	207.55-370.71	55 (29.0)	45 (29.8)	
	≥370.72	45 (23.7)	39 (25.8)	
Alcohol Intake (g/day)	Abstainers	31 (16.2)	23 (15.2)	0.78
	Low	99 (51.8)	84 (55.6)	
	Moderate	61 (32.0)	44 (29.2)	

Dietary Energy Intake (kcal/day)	≤1017.02 1017.03-1388.49 1388.50-1781.56 ≥1781.57	57 (29.8) 52 (27.2) 43 (22.5) 39 (20.5)	35 (23.2) 35 (23.2) 41 (27.1) 40 (26.5)	0.26
Fruit and Vegetable Intake (servings per day)	≤2.9 3.0-4.1 4.2-6.0 ≥6.1	51 (26.7) 53 (27.8) 51 (26.7) 36 (18.8)	37 (24.5) 27 (17.9) 48 (31.8) 39 (25.8)	0.10
Serum Albumin (g/L)	≤40.0 40.1-42.0 42.1-44.0 ≥44.1	53 (28.3) 43 (23.0) 45 (24.1) 46 (24.6)	38 (25.5) 38 (25.5) 42 (28.2) 31 (20.8)	0.68
Serum Folate (nmol/L)	≤26.6 26.7-37.3 37.4-45.3 ≥45.4	47 (25.1) 52 (27.8) 30 (16.1) 58 (31.0)	36 (24.2) 40 (26.8) 31 (20.8) 42 (28.2)	0.73
Serum Cholesterol (mmol/L)	≤4.24 4.25-4.96 4.97-5.75 ≥5.76	58 (31.0) 40 (21.4) 46 (24.6) 43 (23.0)	31 (20.8) 34 (22.8) 38 (25.5) 46 (30.9)	0.15

[1]p-value obtained from a χ^2 test

Table 5. Mean and standard deviation of continuous covariates among quartiles of PhIP

Covariates	**Categories 1 and 2 PhIP≤232.61 ng/day**		**Categories 3 and 4 PhIP>232.61 ng/day**		**p[1]**
	Mean	**SD**	**Mean**	**SD**	
Body Mass Index (kg/cm^2)	27.24	5.32	29.90	6.64	<0.01
Dietary Protein Intake (g/day)	60.03	28.29	65.45	27.37	0.08
Dietary Fat Intake (g/day)	73.06	43.46	76.51	37.33	0.44
Dietary Carbohydrate Intake (g/day)	92.65	1.51	177.06	81.84	0.77
Dietary Fiber Intake (g/day)	26.73	15.32	26.48	14.18	0.88
Serum Lipid Ratio	3.99	1.34	4.43	1.41	<0.01
Serum Calcium (mmol/L)	2.26	0.24	2.27	0.19	0.70
Serum Triglycerides (mmol/L)	1.28	0.81	1.36	0.76	0.38
Serum Vitamin B_{12} (pmol/L)	318.94	180.22	316.27	158.19	0.89

[1]p-value obtained from a t-test

Table 6. Mean and standard deviation of continuous covariates among quartiles of MeIQx

Covariates	Categories 1 and 2 MeIQx≤31.09 ng/day		Categories 3 and 4 MeIQx>31.09 ng/day		p[1]
	Mean	SD	Mean	SD	
Body Mass Index (kg/cm^2)	27.58	5.31	29.59	6.87	<0.01
Dietary Protein Intake (g/day)	62.19	28.70	62.68	27.03	0.87
Dietary Fat Intake (g/day)	75.12	43.09	73.77	37.68	0.76
Dietary Carbohydrate Intake (g/day)	178.35	92.04	171.41	82.09	0.47
Dietary Fiber Intake (g/day)	27.71	15.23	25.09	14.11	0.11
Serum Lipid Ratio	4.0	1.30	4.44	1.47	<0.01
Serum Calcium (mmol/L)	2.26	0.25	2.28	0.17	0.48
Serum Triglycerides (mmol/L)	1.26	0.81	1.39	0.75	0.14
Serum Vitamin B_{12} (pmol/L)	318.64	179.35	316.58	158.54	0.91

[1]p-value obtained from a t-test

Table 7. Mean and standard deviation of continuous covariates among quartiles of DiMeIQx

Covariates	Categories 1 and 2 DiMeIQx≤4.12 ng/day		Categories 3 and 4 DiMeIQx>4.12 ng/day		p[1]
	Mean	SD	Mean	SD	
Body Mass Index (kg/cm^2)	27.49	5.65	29.69	6.43	<0.01
Dietary Protein Intake (g/day)	62.75	28.50	61.87	27.31	0.78
Dietary Fat Intake (g/day)	76.06	42.70	72.39	38.15	0.42
Dietary Carbohydrate Intake (g/day)	177.55	91.88	172.46	82.28	0.60
Dietary Fiber Intake (g/day)	27.35	15.28	25.57	14.10	0.28
Serum Lipid Ratio	4.02	1.35	4.41	1.41	0.01
Serum Calcium (mmol/L)	2.26	0.24	2.28	0.17	0.32
Serum Triglycerides (mmol/L)	1.29	0.81	1.35	0.76	0.54
Serum Vitamin B_{12} (pmol/L)	319.68	180.03	315.07	157.09	0.81

[1]p-value obtained from a t-test

Table 8. Mean and standard deviation of continuous covariates among quartiles of meat mutagenicity exposure

Covariates	Categories 1 and 2 Meat Mutagenicity ≤7179.67 ng/day		Categories 3 and 4 Meat Mutagenicity >7179.67 ng/day		p[1]
	Mean	SD	Mean	SD	
Body Mass Index (kg/cm^2)	27.48	5.67	29.58	6.37	<0.01
Dietary Protein Intake (g/day)	59.59	28.18	65.94	27.41	0.04
Dietary Fat Intake (g/day)	71.93	42.84	77.88	38.14	0.18
Dietary Carbohydrate Intake (g/day)	171.75	91.78	180.16	83.01	0.38
Dietary Fiber Intake (g/day)	26.39	15.30	26.92	14.23	0.74
Serum Lipid Ratio	3.96	1.30	4.45	1.45	<0.01
Serum Calcium (mmol/L)	2.26	0.24	2.28	0.18	0.52
Serum Triglycerides (mmol/L)	1.27	0.80	1.37	0.77	0.26
Serum Vitamin B_{12} (pmol/L)	319.66	182.51	315.40	155.15	0.82

[1]p-value obtained from a t-test

Table 9. Spearman correlation coefficients for dietary exposures to HAAs and meat mutagenicity

$r_{Spearman}$ (p-value)[1]	PhIP	MeIQx	DiMeIQx	Meat Mutagenicity
PhIP	1	0.70 (<0.01)	0.73 (<0.01)	0.90 (<0.01)
MeIQx		1	0.80 (<0.01)	0.85 (<0.01)
DiMeIQx			1	0.83 (<0.01)
Meat Mutagenicity				1

[1]p-value obtained from Spearman rank correlation