Assessment of the overall impact of diet on colorectal cancer in Cambodia, with special emphasis on endogenous and exogenous chemicals

Sokneang In

ParisTech
INSTITUT DES SCIENCES ET TECHNOLOGIES
PARIS INSTITUTE OF TECHNOLOGY

AgroParisTech
INSTITUT DES SCIENCES ET INDUSTRIES DU VIVANT ET DE L'ENVIRONNEMENT
PARIS INSTITUTE OF TECHNOLOGY FOR LIFE, FOOD AND ENVIRONMENTAL SCIENCES

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Evaluation de l'impact global des régimes alimentaires et des composés chimiques endogènes et exogènes sur le cancer colorectal au Cambodge

Assessment of the overall impact of diet on colorectal cancer in Cambodia, with special emphasis on endogenous and exogenous chemicals

Directeur de thèse : **Claude LAMBRE**
Co-encadrants de la thèse : **Mostafa OULD ELHKIM, Philippe VERGER**

Jury

Mme Valérie CAMEL, Professeur, UMR GENIAL, AgroParisTech	Présidente
M. Alain-Claude ROUDOT, Professeur, Université de Bretagne occidentale	Rapporteur
M. Bruno LE BIZEC, Professeur, Laberca, Oniris	Rapporteur
M. Claude LAMBRE, Directeur de recherche, Inserm	Examinateur
M. Mostafa OULD ELHKIM, Chef d'unité toxicologique et microbiologique, Afssaps	Examinateur

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Abstract

From the projection in the future of international bodies, non-communicable diseases such as cancer may increase more than communicable diseases in developing countries. The highlight of this burden may be due to the changing of dietary patterns and lifestyle. Currently, the evolution of food consumption, consumption pattern and colorectal cancer is very worrying worldwide. It is a disease in economically 'developed' populations, and it is the second killer among other cancers; however, its incidence seems lower in poor countries.

As the other developing countries, Cambodia has no system of food survey, no monitoring and control system of chemical substances, and also, there is no control and registration system for cancer. Thus, the objective of this research was to start the first observation of the relationship between dietary pattern and colorectal cancer in Cambodia. The general idea of the study was to identify the dietary patterns, and larger contributors to colorectal cancer in Cambodia, then to translate them into calories, nutrients and bioactive compounds, based on the existing database. Another goal was to assess the difference of dietary habit and cooking methods in the studied population that could lead to the production of colorectal carcinogens such as heterocyclic amines (HAs) and benzo[*a*]pyrene (BaP). In order to create the food consumption database needed for food risk assessment, a food consumption survey was conducted using 24-hour recall and food frequency questionnaire. A dietary assessment of HAs and BaP has been done, in order to establish a hierarchy of the importance of the risk that these substances represent to the health of this population. Dietary exposures to HAs and BaP were obtained by combining food consumption data, obtained from the individual food survey specially designed and carried out during this research, with the contamination data gathered from chemical analysis reported in the recent literature. The observation results have been compared with the toxicological reference values. The results show that dietary patterns in Cambodia have not changed yet to adapt to the Western diet, and contain higher levels of protected nutrients. The exposure to neoformed contaminants (HAs and BaP) was lower than the values reported among other Asian countries, and lowest as compared to the developed countries. The presence of endogenous compounds such as carbohydrates, dietary fibers, calcium and vitamin C, seems to protect the Cambodian population from colorectal cancer. Further research is needed to study the interaction of diet, lifestyle and the genetic background, and other factors as well.

Key words: Cambodia, colorectal cancer, colorectal carcinogens, diet, dietary patterns, nutrients.

Résumé

Dans les pays en voie de développement, les évolutions prévues par les organismes internationaux montrent une progression beaucoup plus importante des maladies chroniques non transmissibles, comme le cancer, que des maladies transmissibles. Actuellement, la majorité des études montre que la tendance à l'augmentation de l'incidence et de la mortalité par cancer colorectal est plus marquée dans les sociétés riches que dans les sociétés pauvres. Dans les pays développés le cancer colorectal est au deuxième rang en ce qui concerne la mortalité par cancer et les changements dans les habitudes alimentaires et le mode de vie sont souvent mis en cause dans son développement. Bien que les données épidémiologiques soient rares, les populations de la plupart des pays asiatiques ne sont pas conscientes du risque grandissant que peut constituer pour eux le cancer colorectal.

Comme les autres pays en développement, le Cambodge n'a pas de système d'enquête de consommation alimentaire, ni de système de surveillance ou de contrôle des substances chimiques, ni de système d'enregistrement, de contrôle ou de dépistage des cancers. L'objectif de cette recherche était de débuter la première observation d'une relation entre régime alimentaire et cancer colorectal au Cambodge. L'idée générale était d'identifier les aliments grands contributeurs des régimes alimentaires de la population cambodgienne, et de les traduire en calories, nutriments et composés bioactifs en se fondant sur les bases de données existantes. Une étude a également été menée sur les comportements de cette population, en ce qui concerne ses habitudes alimentaires et les modes de préparation des aliments susceptibles de produire des substances cancérigènes telles que des amines hétérocycliques (AH) et du benzo[*a*]pyrene (BaP). Pour réaliser ce travail de recherche, une enquête de consommation alimentaire a été effectuée à l'aide d'un rappel sur 24 heures et d'un questionnaire de fréquence alimentaire, afin de créer une base de données dédiée pour servir à l'évaluation des risques. Ensuite, une évaluation de l'exposition aux AH et BaP a été effectuée, afin d'établir une hiérarchie du risque que ces substances posent pour cette population d'étude ; celle-ci a été réalisée en croisant les données de consommation alimentaire, obtenues par le biais d'une enquête de consommation alimentaire individuelle exécutée dans ce travail, avec les données de contamination rassemblées à partir des analyses chimiques reportées dans la littérature scientifique récente. Les résultats ont été comparés avec les valeurs toxicologiques de référence. Ils ont montré que les habitudes alimentaires au Cambodge n'ont pas encore changé pour s'adapter à l'alimentation occidentale, et qu'elles offrent plus de composés protecteurs. L'exposition aux contaminants néoformés (AH et BaP) a été plus faible par rapport aux pays régionaux ainsi qu'aux pays développés. La présence de composés endogènes tels que glucides, fibres alimentaires, calcium et vitamine C semble protéger la population cambodgienne du cancer colorectal. Des recherches supplémentaires sont nécessaires pour étudier les interactions entre l'alimentation, le mode de vie et les facteurs génétiques, ainsi que d'autres facteurs également.

Mots clés : Cambodge, cancer colorectal, carcinogène colorectal, aliment, habitude alimentaire, nutriments

PUBLICATIONS AND COMMUNICATIONS RELATIVE TO THIS WORK

Publications

Hav M, Eav S, Ky V, Cuvelier C, In S, Kong R, Kheang Y, Oung C, Pattyn P and Lem D. 2011. Colorectal Cancer in Young Cambodians. *Asian Pacific J Cancer Prev,* 12, 1-4.

Sokneang IN, Claud LAMBRE, Valerie CAMEL and Mostafa OULDELHKIM. Dietary patterns along with seasonal and regional variability of food consumption in Cambodia. To be summited to Malsian Journal of Nutrition.

Sokneang IN et al. Dietary nutrients intake and micronutrients-defeciency in Cambodia. En cours de rédaction.

Sokneang IN et al. Dietary intake assessment and cooking practice of adult populations with age variation from four differences regions in Cambodia. En cours de rédaction.

Communication

Presentation: Food consumption survey, Experience from Cambodia. Conference-Food Consumption-Exposure Assessment-ILSA-Malaysia. 10-12 October, 2011, Malaysia.

Presentation: Dietary exposure assessment-A case study in Cambodia on Benzo(a)pyrenne. Conference-Food Consumption-Exposure Assessment-ILSA-Malaysia. 10-12 October, 2011, Malaysia.

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1,25(OH)2D3

7,8-DiMeIQx: 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline

ACFs: aberrant crypt focus

A: Ademine

AOM: azoxymethane

APC: adenomatous polyposis gene

AαC: 2-amino-9H-pyrido[2,3-b]indole

BaP: Benzo(a)pyrene

BaPDE: BaP-7,8-diol-9,10-epoxide

BMI: body mass index

b.w: body weight

CDK: cyclin-dependent kinase

CI: confidential interval

COX-2: cyclooxygenase 2

CRC: Colorectal cancer(s)

C: Cystocine

CYP: cytochrome P450

Dietary fibers (DFs)

DiMeIQx: 2-amino-3,4,8- trimethylimidazo-[4,5-f]quinoxaline

DMH: 1,2-dimethylhydrazine

DRIs: Dietary Reference Intakes

DSBs: double-strand breaks

EFSA: European Food Safety Authority

EPIC: European Prospective Investigation into Cancer and Nutrition

FAP: familial adenomatous polyposis

FAO: Food Agricultural Organization

FFQ: Food frequency questionnaire(s)

G: Guanine

Glu-P-1: 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole

Glu-P-2: 2-aminodipyrido[1,2-a:3',2'-d]imidazole

HNPCC: hereditary nonpolyposis colorectal cancer

HAs: heterocyclic amines

IARC: International Agency for Research on Cancer

IDFs: Water-insoluble dietary fibers

IQ: 2-amino-3-methylimidazo[4,5-f]quinoline

LOH: heterozygosity

M: Medium

MDF: mucin depleted foci

MeAαC: 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole

MeIQ: 2-Amino-3,4-dimethylimidazo[4,5-f]quinoline

MeIQx: 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline

MF: mutation frequency

MMR: mismatch repair

MSI: microsatellite instability

NIS: National institute of Statistic, Ministry of Plannings

NOCs: N-nitroso compounds

OR: Odd ration

PAHs: polycyclic aromatic hydrocarbons

PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

R: rare

RDAs: Recommended Dietary Allowances

RNIs: recommended nutrient intakes

RR: relative risk

SCFAs : short-chain fatty acids

SCF: Scientific Committee on Food

SDFs: Water-soluble dietary fibers

SPSS:

SSB: single-strand break

TBM: tribromomethane

TCF: Transcription factor

TGFβ: transcription growth factor-β

T: Thymine

Trp-P-1: 3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole

Trp-P-2: 3-amino-1-methyl-5H-pyrido[4,3-b]indole

WCRF: World Cancer Research Fund

WD: Well done

WHO: World Health Organization

VWD: very well done

Introduction

The human survival is fundamentally related to its diet; nutrition and health are closely connected. It is well known for example that malnutrition, resulting from insufficient uptake of proteins, vitamins and essential minerals can seriously endanger health and a possible way to achieve healthy and well-balanced nutrition is to increase the proportion of plant food in the daily diet by eating at least five portions of fruits and vegetables per day. Development in recent years, relative to both the food itself and the consumer requirements in terms of nutritional quality and safety, have naturally led to what is considered the benefit and risk balance of the food. However, several problems have already been highlighted by the scientific community such as the lack of clear information on the benefits of food compounds, or the lack of studies on the possible adverse health effects of low doses of food chemical contaminants.

Through their diet, humans are exposed to complex mixtures of substances that may be involved in causing, modulating, or preventing colorectal cancer (CRC). Over the world, a wide variation in CRC incidence rate can be noted, with higher rates reported for Westernised countries and lower rates for developing countries. Currently, CRC is the fourth most common cause of cancer deaths worldwide; in the European Union, the burden of CRC is particularly high and it ranks as the second most common cause of cancer deaths. In France, CRC is ranked third of all cancers and it is the second range of mortality. In Cambodia, CRC is in sixth range of cancer incidence and in the fifth range of mortality.

The epidemiology and toxicology studies published to date concluded that CRC development is related to inappropriate lifestyle, behavior patterns and the presence of carcinogens in food. In particular, foods and/or nutrients are strongly related to CRC; for example, red meat and processed meat consumption have frequently been reported to increase CRC risk. The major hypotheses underlying a link between dietary intake and CRC risk could be explained by the presence of several food mutagens such as heterocyclic amines and/or polycyclic aromatic hydrocarbons that can be generated during food cooking and processing. The potentially carcinogenic *N*-nitroso compounds can also be either formed or present endogenously in food. Numerous substances, with either carcinogenic or anticarcinogenic properties, contained in food can interact at the intestinal level and be related to the risk of colorectal tumors.

The topics of the present research work deals with the question of the global impact of dietary intake (including exogenous and endogenous chemical substances) on CRC and seek to provide a better understanding of the relationship between dietary pattern and CRC. To help answer this question,

countries with either high (Western countries, such as European) or low CRC incidence and death (Cambodia) were compared.

Our first objective was to assess CRC incidence and to describe its characteristics in Cambodia. A detailed study on CRC will allow to better understand a real situation of low CRC prevalence in a developing country such as Cambodia. As the most impact factors related to CRC were described in many epidemiological studies to be dietary patterns, dietary habits and cooking practices (with possible formation of carcinogens), a detailed study on dietary patterns and dietary habits has been conducted among the Cambodian population. The main goal of the work described in this thesis was to determine the major factors increasing or preventing CRC development. This was achieved by looking for differences in dietary patterns, dietary habits and assessed exposure to exogenous and endogenous food compounds between the Cambodian population and other populations, based on reported studies in the literature.

Chapter 1 presents the overall research background of the study, especially the potential mechanisms underlying the CRC initiator, promotor and inhibitor effects of substances present in diet (food components and food carcinogens). All mechanisms of action of carcinogens and dietary components on CRC have been collected from literature reviews. We analyzed separately epidemiological and toxicological studies. In epidemiological studies, the relative risk, age, gender, cancer location and follow up study were collected. In the toxicological studies, substance nature, dose, study period, experimental protocol and potential mechanisms of action were collected. This chapter also presents an overview of the currently used methods to assess exposure to colorectal carcinogen.

Chapter 2 gathers information on colorectal cancer in Cambodia. CRC is a common disease in the older population, but it has become increasingly evident that it is also infrequent in the young. CRC is common in the Western world and usually ranks high in incidence and mortality among malignancies in those countries. Two observations have led researchers to look for diet and lifestyle as explanatory factors of risk for CRC. The aim of this Chapter is to describe the epidemiological, clinical and pathological characteristics of CRC in Cambodia, a developing country with a low income for the rural population. Clinical and pathological data on all primary CRC cases diagnosed between 2005 and 2010 were obtained from the database of the Reference Center for Gastro-intestinal Tumours Surgery and Marie Curie Cancer Center, and analyzed with the aim to identify prognostic factors.

Chapter 3 presents data related to dietary patterns and major food sources of energy and nutrients for adult Cambodian population. In this Chapter, detailed information about food consumption surveys using 24-hour recall and food frequency questionnaires (FFQ) are given; results relative to the assessment of dietary intake in Cambodia are also presented. The data of dietary patterns, energy and nutrients intake derived from 24-hour recalls, with 941 subjects (603 females and 337 males, age range 25-65 years old) participating in the two surveys conducted either in wet season or dry season from 2009 to 2010. A specific dietary pattern taking into account differences in cooking practices (especially the use of high temperature) was provided by FFQ. Differences in food consumption patterns between the younger and elderly adults were also evaluated.

This study is the first one to assess the dietary patterns of the Cambodian people in four different regions which cover nearly the whole country, and to describe and analyze the food groups contributing to overall consumption. We tried to assess possible differences in dietary intake between the wet and dry seasons. We also show that differences in consumption patterns among studied population were reflected by differences in common food sources of energy and specific nutrients, some of them being protective factors for CRC. Results of this work will provide the first database of food consumption in the Cambodian population, which is crucial for scientists working in the fields of nutrition, food security and food safety. Such results will be useful in the following chapter in order to assess the exposure of the population to food colorectal carcinogens.

Chapter 4 is related to the assessment of the Cambodian exposure to several food carcinogenic chemicals: three heterocyclic amines (HAs) frequently encountered in meat, using dietary data obtained from FFQ, and benzo[*a*]pyrene (BaP) using dietary data obtained from 24-hour recalls. Exposure assessment methods were developed to link responses to detailed questions about meat preparation and doneness preference to a database of these four carcinogenic compound concentrations in cooked meats prepared in controlled cooking studies reported in the literature. HA and BaP databases have been developed from the earlier published data in the literature, thus the levels of the four carcinogenic compounds under study could be assessed in various types of meat or fish cooked by a number of methods and to varying degrees of doneness. Finaly, the estimated daily exposure and cumulative probability distribution of PhIP, MeIQx, DiMeIQx and BaP have been calculated. In addition, dietary and cooking practices of several specific foods have been characterized and explored in order to identify the potential sources of carcinogenic HAs in the Cambodian diet, which could suggest a possible association with the high incidence rate of CRC amount young Cambodian.

Chapter 5 is a general discussion on dietary patterns, colorectal carcinogens and CRC. A comparison of dietary habits and dietary intakes, further converted into energy, macronutrients and micronutrients intakes, is discussed followed by the assessment of exposure to potential chemical substances formed during cooking. Results of exposure assessment using a deterministic approach were compared with data from the literature.

Finally, the general conclusion and some future perspectives are presented.

Chapter 1. Colorectal cancer: etiology, incidence, and role of diet

1. Generality on colorectal cancer incidence

The GLOBOCAN 2008 series published by the International Agency for Research on Cancer (IARC), showed that the colorectal cancer (CRC) is the third most common cancer in men and the second in women (Ferlay *et al.,* 2010). Currently, CRC is the fourth most common cause of cancer deaths worldwide. Almost 60% of the cases occur in developed regions and the incidence rates of CRC vary widely between countries and regions. In the European Union, the burden of CRC is particularly high: it ranks as the second most common cause of cancer deaths (Ferlay *et al*., 2010; Ferlay *et al*., 2007). Incidence rates vary 10-fold in both sexes worldwide, the highest rates being estimated in Australia/New Zealand and Western Europe, the lowest in Africa (except Southern Africa) and South-Central Asia, and are intermediate in Latin America (**Fig. 1.1**). Incidence rates are substantially higher in men than in women (overall sex ratio of the age-standardized incidence rate1.4:1) (Ferlay *et al*., 2010; Pisani *et al*., 2002; Potter., 1999).

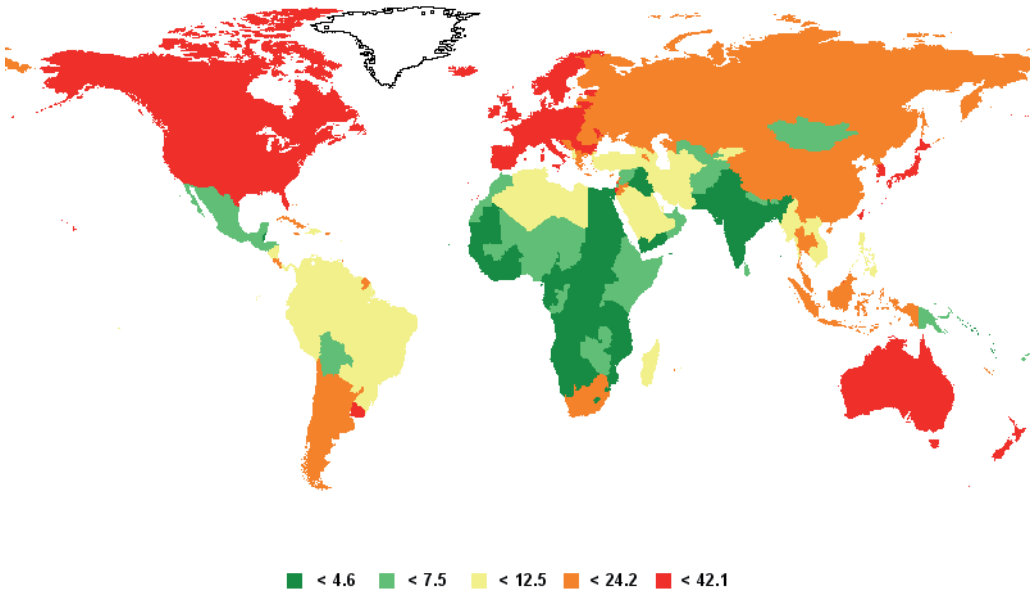


Figure 1.1. Estimated age-standardized incidence rate per 100 000 colorectal cancers: both sexes, all ages (GLOBOCAN, 2008).

However, many Asian countries including, Japan, China and South Korean have experienced a two to four fold increase in the incidence of CRC in recent decades. The rising trend in incidence and

mortality from CRC is more striking in affluent than in poorer societies, and differs substantially among ethnic groups. Although changes in dietary habits and lifestyle are believed to be the main reasons underlying the increase, interactions between these factors and genetic characteristics of the Asian populations might also have a pivotal role. Non-polypoidal (flat or depressed) lesions and colorectal neoplasm arising without preceding adenoma (de *novo* cancers) seem to be more common in Asian than in other populations (Sung *et al*., 2005; Tamura *et al*., 1996). As observed for incidence, mortality rates are lower in women than in men, except in the Caribbean. There is less variability in mortality rates worldwide (six fold in men, five fold in women), with the highest mortality rates in both sexes estimated in Central and Eastern Europe (20.3 per 100 000 for males, 12.1 per 100 000 for females), and the lowest in Middle Africa (3.5 and 2.7 per 100 000 for males and females, respectively) (Ferlay *et al*., 2010).

2. Pathogenesis of colorectal cancer

2.1. The large intestine and the crypt

The large intestine or large bowel is the second-to-last part of the digestive system, the final stage of the alimentary canal being the anus in vertebrate animals. Basically, the colon in a living human adult is an approximately 1.5 meter long tube in which water, sodium, and other minerals are absorbed from the remaining indigestible food matter, and then useless waste materials pass from the body. This tube is composed of three layers (**Fig. 1.2**). The outer layer is made of a smooth muscle that facilitates peristalsis. The middle layer, or sub-mucosa, consists of stromal tissue and the inner layer of this tube is an epithelial layer, like the epidermal cells of the skin, which forms a barrier between the body and the outside world. The epithelial layer is folded to form invaginations, or crypts, which are embedded in the stromal tissue (Booth *et al*., 2000). The epithelium plays a crucial role in the maintenance of intestinal homeostasis (Takahashi *et al.,* 2009) and actively samples resident bacteria, pathogens and other antigens (Kagnoff *et al*., 1997; Rakoff-Nahoum *et al*., 2004). The epithelium is covered by mucus that protects the mucosal surface by limiting pathogen access. Mucus is mainly composed of complex glycoproteins called mucins, which are encoded by various mucin genes (Corfield *et al*., 2000; Gaudier *et al*., 2006). Intestinal epithelial cells secrete many mediators involved in immune responses to potentially pathogenic organisms, including antibacterial peptides such as defensins (Vora *et al*., 2004), mucins including MUC3 (Corfield *et al*., 2000), and chemokines and cytokines such as interleukin (IL)-8 (Stadnyk, 2002).

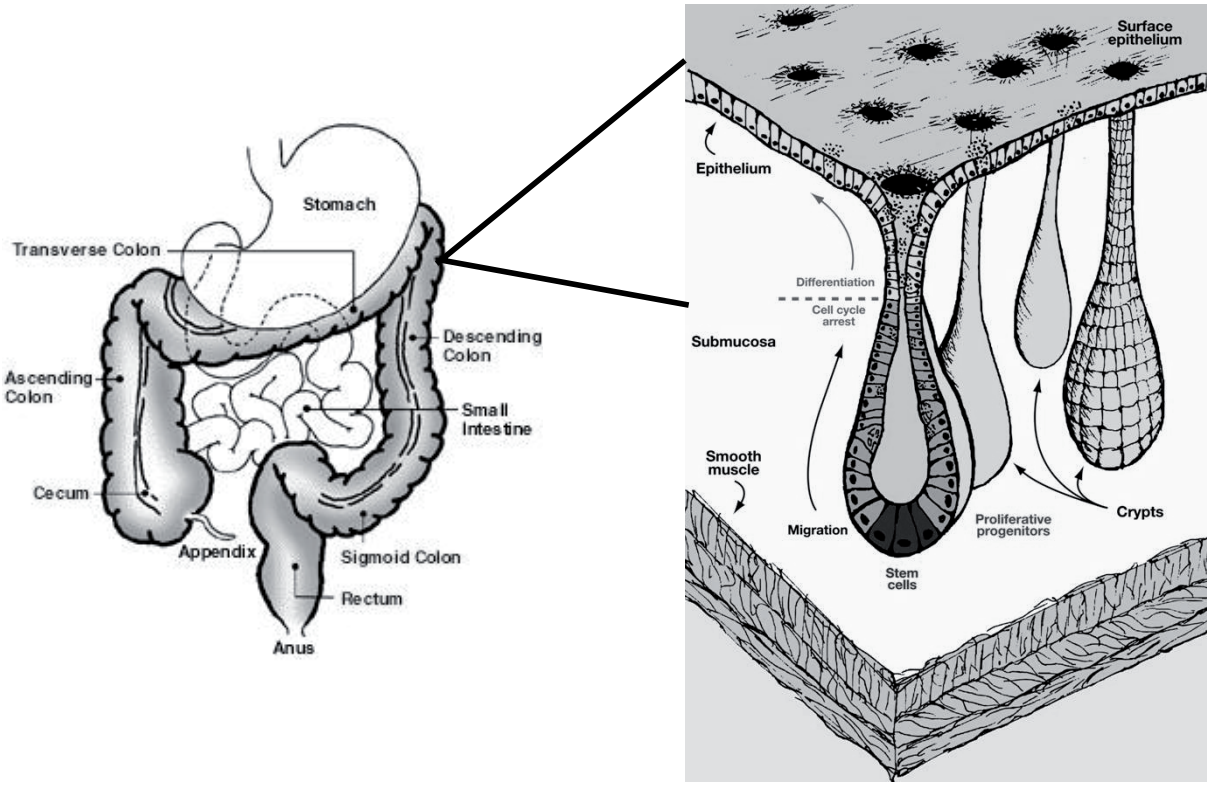


Figure 1.2. Schematic overview of the human intestinal system, along with the structure of the large intestine (de Vogel, 2006).

The mammalian intestinal mucosa, with its distinctive polarity, high rate of proliferation and rapid cell migration, is an excellent model system to study proliferative hierarchies and the regulation of cell division, differentiation and cell death. Each crypt contains a few lineage ancestral stem cells (the 'ultimate stem cells'), which are located at the base of the crypt. However, there are other potential stem cells within the early lineage, and many rapidly proliferating transit cells with no stem cell capabilities. The lower half of the crypt is occupied by proliferative cells. These cells actively migrate in a continuous pattern from the bottom compartment upward to the luminal surface of the colon (Potten, 1998). After the cells exit the proliferative compartment they differentiate into enterocytes (absorptive cell lineage) or secretory cells. Secretory cells in the colon encompass mainly goblet cells secreting protective mucins or enteroendocrine cells involved in the secretion of different hormones (Potten *et al*., 1997). These processes imply that only fully differentiated epithelial cells are found at the top of the crypts and the surface epithelium of the colon. In time, these terminally differentiated surface epithelial cells are detached from the basal membrane and shed intact into the luminal contents (Anexe I). An important characteristic of the epithelial cells is their capacity to

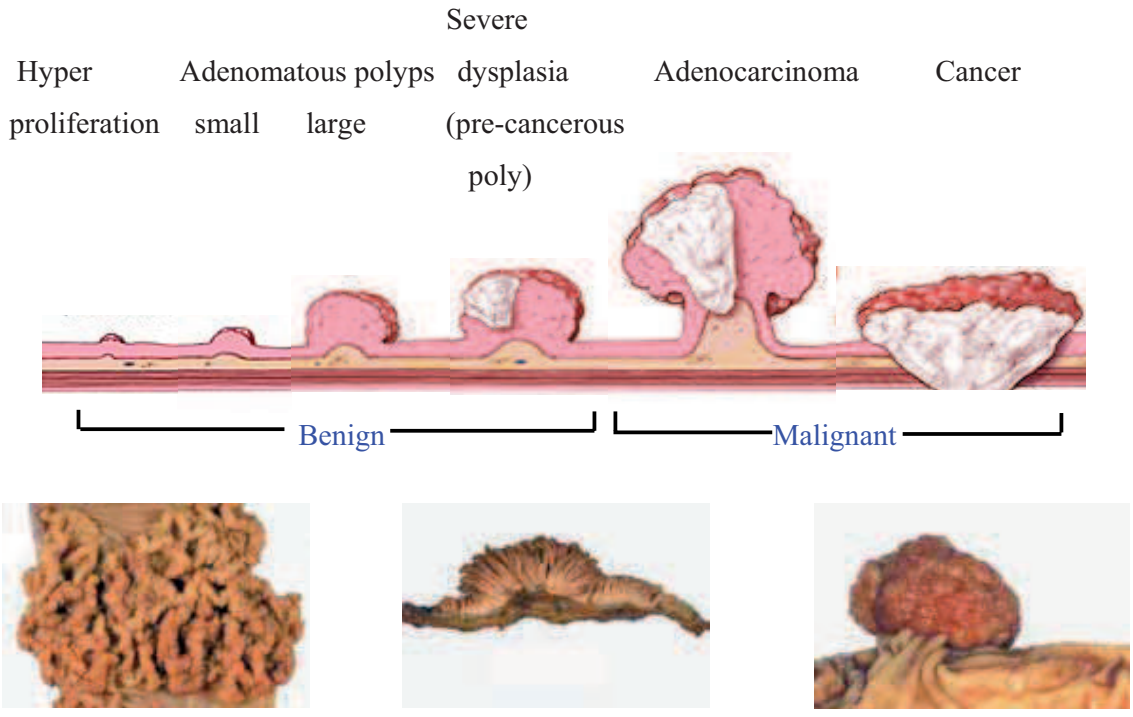
renew themselves. Normal homeostasis in the crypt implies that the rate of crypt cell regeneration is tightly coupled with loss of surface epithelial cells (Podolsky, 1993; Potten, 1998). A consequence of the high self-renewing rate of epithelial cells might be a high susceptibility to malignant transformation, as this can only occur in dividing cells.

2.2. Histological type of colorectal cancer

Colorectal tumor locations can be divided into three groups: (i) right colon, which includes appendix, caecum, ascending colon, hepatic flexure colon and transverse colon; (ii) left colon, which includes splenic flexure colon, descending colon and sigmoid colon; and (iii) rectum, which is below the level of the sacral promontrium (Oya *et al*., 2003). CRC is a complex multi-step process, thought to be caused by an accumulation of multiple genetic mutations resulting in a transformed phenotype. These multiple stages lead to transformation of normal colonic epithelium into the development of pre-neoplastic lesions, followed by early and advanced adenomas to invasive carcinoma (**Fig. 1.3**) (Fearon *et al.,* 1990; Fearon *et al*., 1992). Initiation and progression of tumorigenesis from a normal colonic mucosa into a frank carcinoma and metastasis is generally associated with characteristic histopathological and morphological features. Adenocarcinoma is the most common colorectal tumour type (Treanor *et al*., 2007), being divided into several subtypes: mucinous adenocarcinoma, signet-ring cell carcinoma, small cell carcinoma, squamous cell carcinoma, adenosquamous carcinoma, medullary carcinoma, undifferentiated carcinoma (Hamilton *et al*., 2000; Treanor *et al*., 2007). At least 50% of the tumour must be mucinous in order to make this diagnosis. Mucinous adenocarcinomas are associated with microsatellite instability (Treanor *et al*., 2007).

The majority of CRC arises from pre-existing adenomatous polyps. The two most common types of polyps found in the large intestine are hyperplastic polyps and adenomas or adenomatous polyps. Histologically, hyperplasic polyps contain an increased number of glandular cells with decreased cytoplasmic mucus, but lack nuclear hyperchromatism, stratification, or atypia (Tsai *et al*., 1995). Adenomatous nuclei are usually hyperchromatic, enlarged, cigar-shaped, and crowded together in a palisade pattern (Rubin *et al*., 2003). Adenomas are classified as tubular or villous. Histologically, tubular adenomas are composed of branched tubules, whereas villous adenomas contain digitiform villi arranged in a frond. Tubulovillous adenomas contain both elements (Hamilton *et al*., 2000).
Adenomatous polyps originate from the epithelium and develop as a result of abnormal cell proliferation (dysplasia). These types of polyps are very common (Bafandeh *et al.,* 2006) and may occur in 50% of people over the age of 60 years. The risk of colon cancer markedly increases with increasing number of adenomatous polyps (Bussey, 1975; Heald *et al*., 1975). It is more frequently

found in the distal part of the colon and the rectum; therefore it is often referred to as colorectal adenomas (Bafandeh *et al.,* 2006; Ikeda *et al.,* 1999). Adenomatous polyps can be of various sizes, ranging from small pedunculated lesions to large sessile polyps. These are neoplasic polyps, meaning that they have the potential to become malignant cancerous (Bond, 2000a; 2000b). This malignant potential increases with severity of the dysplasia and with increasing size of the polyp. A strong familial predisposition is seen with adenomatous polyposis syndromes. Adenomatous polyps associated with hereditary polyposis syndromes carry a significantly higher risk of CRC (Morson *et al*., 1976). Having a first-degree relative with familial polyps increases the risk for adenomatous polyps by four-fold.



A. Sessile villous adenoma.

B. Section through a villous adenoma.

C. Tubular adenoma of colon.

Figure 1.3. Multi stage of CRC development from benign to malignant, and histological types of adenoma (adapted from Hamilton *et al*., 2000).

Initiation and progression of tumorigenesis from a normal colonic mucosa into a frank carcinoma and metastasis is generally associated with characteristic histopathological and morphological features. In CRC, the adenoma-carcinoma sequence is initiated with the emergence in the intestinal epithelium of small lesions of irregular glandular architecture, called aberrant crypt focus (ACF), which is the earliest morphological precursor of epithelial neoplasia (Hamilton *et al*., 2000). ACF

initially identified on the colonic musoca of rodents exposed to colorectal carcinogens have long been regarded as preneoplastic or precancerous lesions (Bird, 1987; Bird *et al*., 1989); they have been also recognized in human (Pretlow *et al*., 1991; Takayama *et al*., 1998). ACF was divided into two types holding differential histological features (Nucci *et al.,* 1997), with distinct potential for progression to colon cancer (**Fig. 1.4**).

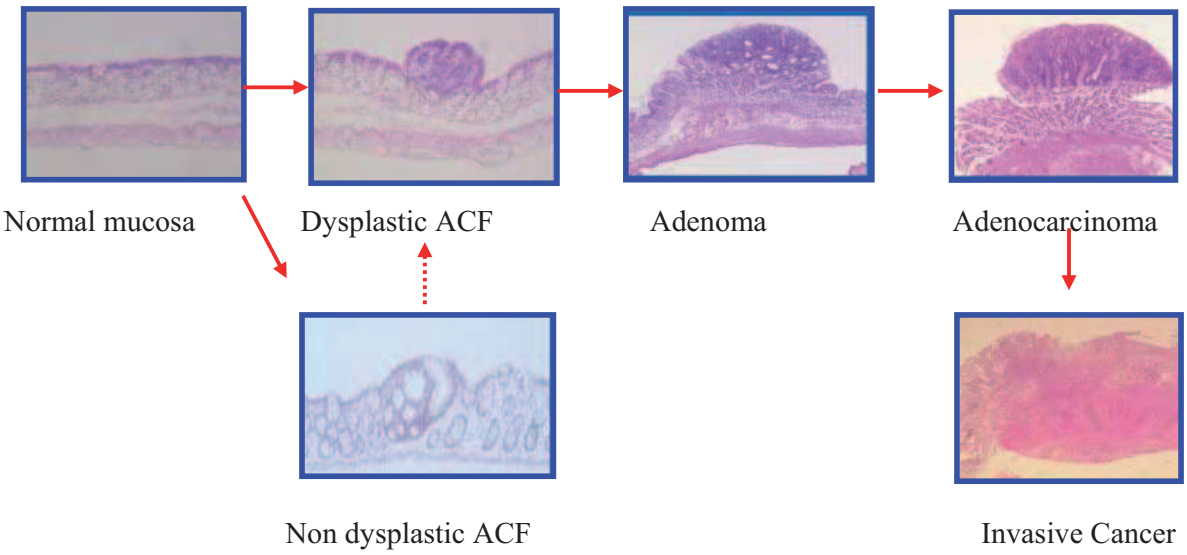


Figure 1.4. Multi-stage model of rat colon cancer induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), a carcinogenic compound; aberrant crypt focus (ACF), a candidate preneoplastic lesion of the colon, is induced shortly after exposure to PhIP (Bird, 1987; Bird *et al*., 1989).

The dysplastic or unicrystal ACF type has an increased likelihood to progress to cancer, and it is thought to arise due to bi-allelic inactivation of the APC tumor suppressor gene (Lamlum *et al*., 2000; Smith *et al.,* 1994). ACFs, identified in whole-mount preparations of colonic mucosa in rodents and also recognized in human colon, are now frequently used as effective surrogate biomarkers for experimentally detection of chemopreventive agents against CRC (Mori *et al*., 2004; Pretlow *et al*., 1991). ACFs are defined as single or multiple crypts that have altered luminal openings, exhibit thickened epithelia, and are larger than adjacent normal crypts (Bird *et al*., 1989). It has been proven that the number of crypts/focus increases with time after carcinogen treatment, and that ACFs increased the cell proliferation in rodents (Pretlow *et al*., 1992; Pretlow *et al*., 1994). ACFs have been confirmed in hamsters as well as in rats and mice, and basically are observed in the colons of all animals exposed to colon specific carcinogens (Feng *et al*., 1996; Paulsen *et al*., 1996).

2.3. Genetic and epigenetic changes, alteration mechanism of CRC

By the age of 70, approximately 50% of men and women from western industrialized societies are likely to having developed CRC (Kinzler *et al*., 1996). Traditionnally, CRC is divided into sporadic and familial (hereditary) forms (Jass *et al*., 2002; Hemminki *et al*., 2002), and represents a complex disease, whose development is determined by different combinations of genetic and environmental factors (Potter, 1999). More than 80% of CRC occur sporadically, which means that affected patients do not have a family history of colon cancer (Fearnhead et al., 2002), with approximately 70% of CRC attributed to an inappropriate diet (Doll *et al*., 1981; Willett, 1995). Apart from the effects of exogenous factors, CRC risk may be influenced by hereditary factors or a combination of genetic and environmental factors. Patients with familial risk make up approximately 20% of all patients with CRC, whereas about 5% of the total annual burden of CRC is caused by autosomal dominant genetic factors. Two of the main forms are familial adenomatous polyposis (FAP) and hereditary nonpolyposis CRC (HNPCC) (Kinzler *et al*., 1996; Shanmugathasan *et al.,* 2000). FAP is caused by a germline mutation in the adenomatous polyposis gene (APC), while HNPCC is associated with germline mutations in DNA mismatch repair (MMR) genes (Kinzler *et al*., 1996). Somatic inactivation of the MMR genes in sporadic microsatellite instability (MSI) CRC occurs either by somatic mutation and loss of heterozygosity (LOH) events at the major MMR genes (namely MSH2, MLH1, and MSH6) (Peltomaki, 2003) or, more frequently, by epigenetic silencing through promoter methylation of the MLH1 gene (Jones *et al*., 2002). Somatic inactivation of the MMR system additionally gives rise to approximately 15% of sporadic colon cancers and these alterations provide growth advantage and lead to clonal expansion of distorted cells. Importantly, MSI and subsequent target gene mutations appear to occur throughout the adenoma-to carcinoma progression (Fearon *et al*., 1990; Ilyas *et al*., 1999; Lynch *et al.,* 2003).

- **APC, K-RAS, transforming growth factor β/SMAD, p53 pathways**

A model was proposed to explain the genetic basis of colorectal neoplasia that includes several salient features. Genomic instabilities caused through mutation or lack of DNA damage can lead to cancer transformation (Vogelstein *et al*., 1988). There are two major possible processes that may be responsible for inducing genetic mutation in colonocytes: the direct action of mutagens, causing genetic damage, or increased proliferation causing more errors in DNA repair. A cellular accumulation of these genetic mutations will likely to undergo full malignant transformation (Fearon *et al.,* 1990). For CRC, the evolution of normal epithelial cells to benign adenomas and malignant carcinomas usually follows a multi-step progression model of histological stages and occurrent genetic and epigenetic changes. According to Fearon and Vogelstein (1990), a genetic model for

colorectal tumorigenesis is presented in **Figure 1.5**. In the genetic alterations and pathways involved in colorectal tumorigenesis, the Vogelstein model has shown that the key genetic events can be grouped into two categories: 80–85% of tumors exhibit chromosomal instability, 15-20% of tumors being characterized by microsatellite instability (Fearon *et al*., 1992; Ilyas *et al*., 1999; Lengauer *et al*., 1998). In the first pathway, chromosomal instability is characterized by both numerical and structural changes cumulatively referred to as aneuploidy (Grady *et al*., 2002; Ilyas *et al*., 1999); it has now been established that alterations of the WNT/β-catenin, K-RAS, TGFβ and TP53 pathways characterize the adenoma-carcinoma sequence in most hereditary and sporadic CRC cases (Kinzler *et al*., 1996). The second pathway, associated with microsatellite instability, occurs in 15–20% of sporadic CRC cases (Lengauer *et al*., 1998). The hallmark of microsatellite instability is represented by small deletions or insertions in stretches of short repetitive DNA sequences (microsatellite repeats) distributed throughout the genome (Ionov *et al*., 1993; Thibodeau *et al*., 1993).

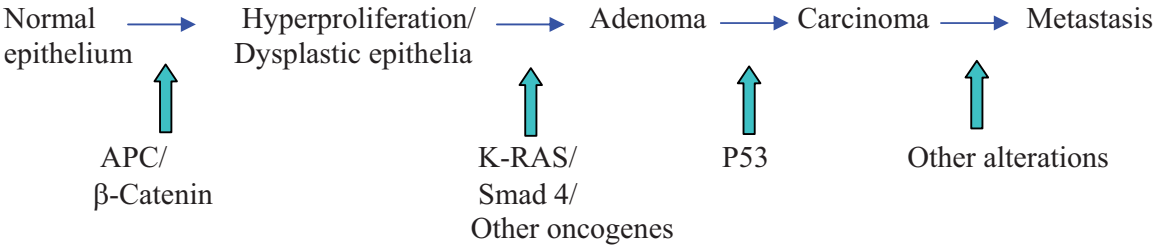


Figure 1.5. A genetic model for colorectal tumorigenesis (adapted from Fearon and Vogelstein, 1990).

APC, K-RAS and P53 mutations have been shown to be significant genetic alterations in the development of CRC (Kinzler *et al*., 1996; Powell *et al*., 1992; Vogelstein *et al*., 1988); they have been demonstrated in ACFs (Janssen *et al.,* 2002; Smith *et al.,* 1994). In sporadic colorectal carcinogenesis, assuming the biological implication of ACF as a precursor of adenomas, there is a route where K-RAS mutation mainly occurs during the formation of ACFs, which then become adenomas wherein APC mutation occurs. In FAP, however, somatic mutation of APC predominantly occurs during ACF formation, followed by K-RAS mutation (Takayama *et al*., 2001).

Initiation, the first stage of colorectal carcinogenesis, is often associated with mutation in the adenomatous polyposis coli (APC)/β-catenin pathway or mismatch repair genes. Both genes play a pivotal role in the regulation of mucosal proliferation and are classic tumor suppressor genes. In sporadic CRC, mutations in APC initiate the majority of the tumors (Kinzler *et al*., 1996): mutations in the APC gene on chromosome 5q21 locus are found in 60 to 80% of sporadic CRC and adenomas

(Narayan *et al*., 2003). APC is known to regulate β-catenin-TCF or Wnt signaling. The APC protein interacts in a complex with β-catenin, glycogen synthase kinase 3β and axin to regulate the level of β-catenin by targeting β-catenin for degradation by the ubiquitination-proteosome pathway (Augenlicht *et al*., 2002). In the absence of functional APC, β-catenin levels rise, enabling it to form an active complex with the transcription factor TCF-4 (van Es *et al*., 2001). The β-catenin-TCF-4 transcription complex targets the expression of several important genes, including cyclin D1 (Shtutman *et al.,* 1999; Tetsu *et al*., 1999) and c-myc (He *et al*., 1998), the latter in turn activating the transcription of the cyclin-dependent kinase, CDK4 (Hermeking *et al*., 2000).

The APC and mismatch repair genes were found to initiate FAP and HNPCC, the two main autosomal dominantly inherited CRCs, respectively (Kinzler *et al*., 1996). Mutations in the APC gene are responsible for the disease FAP, where patients develop numerous benign tumors of colon. Some of these tumors will progress to cancer if not removed surgically. Loss of heterozygosity (LOH) and bi-allelic APC gene mutations are observed in near 70–80% of FAP and sporadic colorectal tumors regardless of histological stage (Miyoshi *et al*., 1992; Powell *et al*., 1992). Loss of APC function deregulates the Wnt/β-catenin signaling pathway, the main rate-limiting step in CRC initiation (Fodde *et al.,* 2001). Wingless/Wnt signaling pathway with subsequent expression of genes that favor cell growth is one of the central tumor-promoting effects of APC mutation. The central player in this signaling cascade is β-catenin (CTNNB1), a protein with variable intracellular localization at the membrane, cytoplasm, and nucleus of epithelial cells. APC mutations disrupt the association of APC with β-catenin, resulting in excessive amounts of β-catenin and over activation of the Wnt signaling pathway (Arends *et al*., 2000; Grady *et al*., 2002).

A large number of cellular oncogenes have been identified in human tumors, including colon cancer. One of the most prominent protooncogenes in colon carcinogenesis is a member of the RAS family of genes, K-RAS. The K-RAS gene is the most commonly mutated RAS family member in colon cancer, although N-RAS mutations are also observed in a small percentage of colon cancers (Fearon, 1995). K-RAS mutations appear to occur after APC mutations are formed, and are associated with advanced adenomatous lesions. Mutational activation of the K-RAS2 oncogene is found in up to 50% of all CRCs (Bos *et al*., 1987; Vogelstein *et al*., 1988). K-RAS pathways transduce signals from extra-cellular growth factors to regulate progression through the cell cycle and proliferation. K-RAS mutations are associated with up-regulation of DNA methyltransferase, CCND1 and gastrin (GAST) (Arends *et al*., 2000; Grady *et al*., 2002). As in other tumors, the K-RAS mutations observed in colon cancer almost always affect codons 12, 13 and 61. Codon 12 is the most commonly mutated in

colorectal cancer and usually undergoes a missense mutation (Vogelstein *et al*., 1988). K-RAS mutations have been found in 37–41% of colon carcinomas and appear to occur relatively early in colon-cancer formation (Arber *et al*., 2000; Bos *et al*., 1987). In the absence of K-RAS2 mutations, 20% of CRC contain activating mutations in BRAF (Ionov *et al*., 1993; Lengauer *et al*., 1998); activating mutations in K-RAS2 and BRAF are generally associated with adenoma growth and progression (Rajagopalan *et al.,* 2002; Vogelstein *et al*., 1988). In sporadic colorectal carcinogenesis, assuming the biological implication of ACF as a precursor of adenomas, there is a route where K-RAS mutation mainly occurs during the formation of ACF, which then become adenomas wherein APC mutation occurs. In FAP, however, somatic mutation of APC predominantly occurs during ACF formation (APC mutation occurs in 100% of FAP-associated ACF), followed by K-RAS mutation (Takayama *et al*., 2001; Vivona *et al*., 1993), but APC mutation is rare in sporadic ACF (Lance *et al*., 2008; Takayama *et al*., 2001).

Tumor progression to late adenomas and early carcinomas is accompanied by alterations of the transcription growth factor-β (TGFβ) signal transduction pathway. TGF-β is a multifunctional cytokine that can induce growth inhibition, apoptosis and differentiation in intestinal epithelial cells (Arends *et al*., 2000; Grady *et al*., 2002). The vast majority of CRCs carry inactivating mutations in at least one component of the TGFβ pathway such as the TGFβ receptor 2 (TGFβR2) (Fearon *et al*., 1992) and the SMAD2 and SMAD4 genes (Bird, 1987; Fearon *et al*., 1990) thus affecting angiogenesis, cell proliferation and differentiation (Bird *et al*., 1989; Pretlow *et al*., 1991). Mutational inactivation of SMAD2 and SMAD4 has been observed in a high percentage of pancreatic cancer and in 5–10% of colon cancers. Next to deleted in Colorectal Carcinoma, these two tumor suppressor genes are the targets of 18q loss of heterozygosity (LOH) (Arends *et al*., 2000; Grady *et al*., 2002).

The last stage of carcinogenesis is progression. It involves additional growth of the ademona and invasion of the basement membrane. Loss of P53, a tumor suppression gene, is the major genetic change associated with progression of tumor growth to carcinoma (Arends *et al*., 2000; Grady *et al.,* 2002). P53 is a transcription factor with tumor suppressor activity, mutated in 50% of primary human tumors, including tumors of the gastrointestinal tract (Somasundaram, 2000). Bi-allelic inactivation of the P53 tumor suppressor gene earmarks the malignant transformation from adenoma to carcinoma in at least 45% of all CRC (Nucci *et al*., 1997; Takayama et al., 1998). The P53 protein is a transcription factor that normally inhibits cell growth and stimulates cell death when induced by cellular stress; its inactivation has consequences for the maintenance of genome integrity in intestinal tumor cells. The P53 gene is known to arrest cell cycle progression, increase apoptosis and allow the

DNA to be required. In cancer cells with mutated P53, cell proliferation is no longer controllable; resulting in inefficient DNA repair and the emergence of genetically unstable cells (Soussi, 2000). The best-characterized tumor suppressors are P53 on chromosome 17p13.1, which shows a LOH frequency of 75% in CRC, Smad4/DPC4 (deleted in pancreatic cancer locus 4) on chromosome 18q21, and APC on chromosome 5q21-22 (Baker *et al*., 1989; Nishisho *et al.,* 1991). Most frequent genomic losses in CRC are found at chromosome 18q21 (Fearon *et al*., 1990), and most frequent gains are found at 20q13 as determined by comparative genomic hybridization (Korn *et al*., 1999).

- **DNA-adducts, chromosome break**

It is both an old and contemporary subject to understand the roles of the two mechanisms for carcinogenesis, genetic alterations with changes in DNA sequences and epigenetic alterations without changes in DNA (Jones *et al*., 1999; Łuczak *et al*., 2006). Solid evidence has accumulated for base-pair change, deletion, insertion, recombination and amplification of oncogenes, tumor suppressor genes and genes related to metastasis and invasion (Sugimura *et al*., 1992). The genetic alteration mechanism has also been supported by the presence of many endogenous and exogenous chemicals, being able to cause changes in DNA sequences (Barrett, 1993). The covalent binding of xenobiotics or their reactive metabolites to DNA is believed to initiate this chemical carcinogenesis. DNA-adducts are one of the mechanisms through which genotoxic agents may cause mutation and lead to induction of cancer. A DNA-adduct is the covalent linking of an abnormal radical to DNA with the potential for cancer formation; for example the main carcinogens include heterocyclic amines (HAs), polycyclic aromatic hydrocarbons (PAHs) and N-nitrosamines (Guttenplan *et al*., 2003**;** Lin *et al*., 1992; Suh et al., 1995). The misreplication of DNA-adducts can lead to malignancy. Alkylating or oxidative agents cause the majority of documented adducts. Recently, there have been speculations that these adducts are linked to greater CRC risk (Lewin *et al*., 2006). O6-methylguanin DNA-adducts are formed between nitrosated glycine derivatives and DNA (Lewin *et al.,* 2006; Shuker *et al.,* 1997). Furthermore, recent *in vitro* studies have demonstrated that formation of O6-methylguanine DNA-adducts lead to mutation in P53 similar to those observed in human gastrointestinal tract tumors (Gottschalg *et al*., 2007). A study in human volunteers has shown that dietary red meat consumption led to significantly higher O6-methylguanine DNA-adducts in colonocytes than white meat consumption (Lewin *et al*., 2006). Collectively, these data suggest that adduct formation is a potential mechanism for carcinogenesis which can be modulated by diet to increase risk. DNA repair is an important process to maintain the integrity of DNA sequence. It seems to contribute to tumorigenesis and is also a mechanism for tumor resistance to chemotherapy (Brabec *et al*., 2002; Christmann *et al*., 2003). Cellular repair of DNA is composed of several distinct

pathways, which includes reversion repair, base excision repair, nucleotide excision repair, MMR, and DNA double-strand break repair (Christmann *et al*., 2003; Kunkel *et al*., 2005; Rosell *et al*., 2003). The DNA repair pathway is under the regulation of multiple cellular processes, which can be crucial determinants to the fate of tumor cells exposed to DNA-damaging agents: resistance or apoptosis. It has been known that P53 is a potent mediator of cellular responses against genotoxic insults and exerts its effect on the DNA repair pathway in sequence-specific DNA binding mode primarily at the transcriptional level (Adimoolam *et al*., 2003; Zhou et al., 2003; Fei et al., 2003).

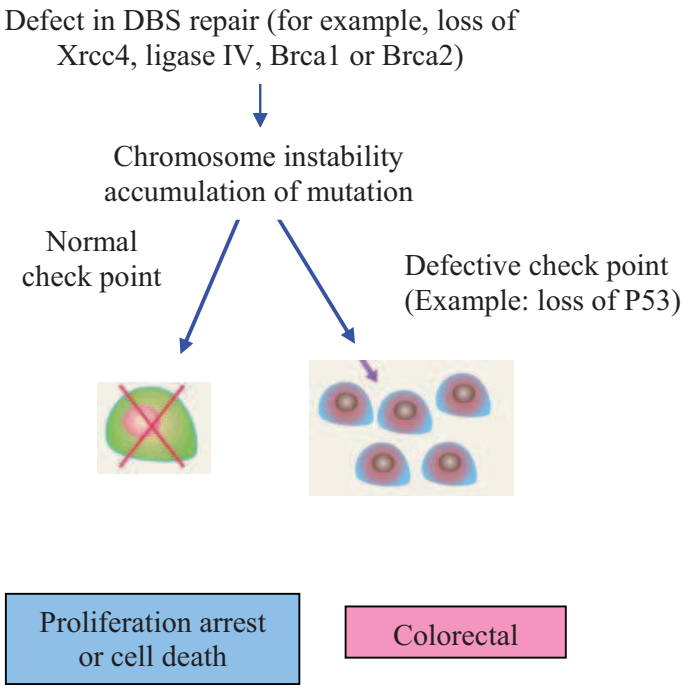


Figure 1.6. Double-strand break repair gene involved in colorectal cancer (adapted from Khanna *et al*., 2001).

Chromosome breaks are documented measures of DNA damage, with single-strand DNA break (SSB) being one of those measured frequently. SSB arises consistently from DNA base damage and must be repaired to maintain genomic stability. If the repair process is hindered, this can lead to double-strand breaks (DSBs) during DNA replication, and can result in chromosome instability and cell death (**Fig. 1.6**). DSBs are considered to be one of the most dangerous forms of cellular genomic damage. DSBs differ from other types of DNA lesion in that both strands act as a template for repair (van Gent *et al.,* 2001; Khanna *et al*., 2001). In the human CRC, genetic polymorphism of DNA double-strand break repair gene may contribute (Bau *et al*., 2010).

Cell proliferation

Colonic epithelial cells in man begin to develop some of the characteristics of malignant cells while still appearing normal on conventional morphological examination. Cell proliferation is often associated with carcinogenesis in rodents and humans because it can increase mutation rates or help to fix spontaneous or induce mutations. In **Figure 1.7**, the possible role of an increased cell proliferation as a risk factor for colon cancer is shown, according to Lipkin (1974). The cells pass through specific phases during which they express increasingly abnormal proliferative characteristics. As a Phase 1 proliferative lesion develops, colonic epithelial cells do not repress DNA synthesis during their maturation, and begin to develop an enhanced ability to proliferate. The over-all kinetics of cell proliferation remains normal, without a net retention or accumulation of cells in the mucosa. As a Phase 2 proliferative lesion forms, the cells begin to develop properties that enable them to be retained in the mucosa in increasing numbers. The over-all kinetics of cell proliferation becomes abnormal, and net retention and accumulation of cells begin to be observed (Lipkin, 1974).

It is widely believed that stem cells are the relevant target cells for colonic cell transformation. Stem cell daughters proliferate in the lower part of the crypt, move in a tight cohort toward the upper crypt regions, gradually lose their capacity to divide and finally acquire the differentiated phenotype (Hall *et al*., 1994; Williamson, 1978). Evidence are presented that a proliferative transit daughter cell acquiring a mutant adenomatous polyposis coli gene during upward migration from the crypt base can develop retention abnormalities and permanence in the crypt, thus qualifying as a transformed clone which is retained in the colonic epithelium cell. Such cells will produce in time dysplastic crypts that will branch as the lesions expand from the bottom of the crypt (Lamprecht *et al.,* 2002). The changes are accompanied by differentiation-specific molecular errors, resulting in the abnormal persistence of metabolic pathways leading to enhanced DNA synthesis. These proliferative cellular lesions arise in hereditary familial polyposis in man, in individuals in the general population who develop isolated colonic neoplasms, and in rodents administered a chemical carcinogen, suggesting that they are major steps on a final common molecular pathway leading to malignant transformation (Lipkin, 1974).

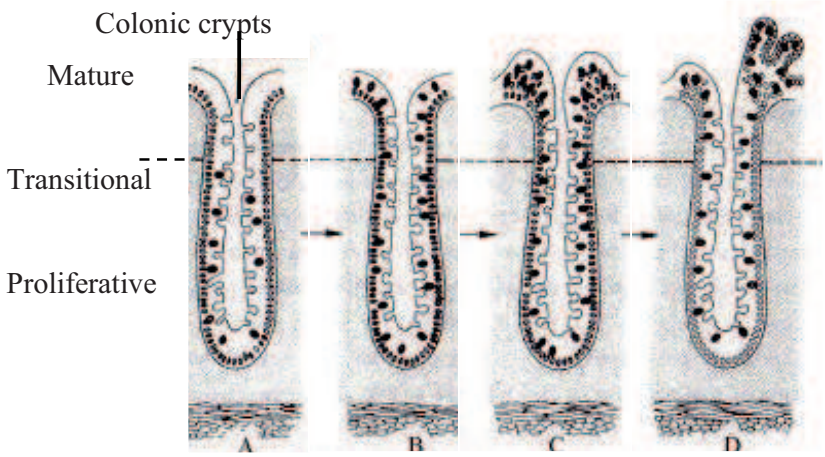


Figure 1.7. Diagram of proliferative, transitional, and mature cell zones within colonic crypts; this cell proliferation plays a role in the development of colorectal cancer according to Lipkin (1974). A- Normal colonic crypt; dark spots represent cells undergoing DNA synthesis. B- Proliferative zone expands to higher one-third of crypt. C-The zone of maximum proliferative activity shifts to the top of the crypt; cells accumulate in the musoca. D-Adenoma appears.

3. Influence of dietary intake on colorectal cancer development

Increased CRC risk is associated with both dietary factors (intake of excess fat, sugar and alcohol; low amount of vegetables and fiber) and lifestyle factors (low physical activity, high body mass index and smoking) typical of Western societies. Based on epidemiological studies, 70 to 90% of CRC mortality can be attributed to dietary factors (Doll *et al*., 1981; Willett, 1995), so that it remains clear that CRC could largely be prevented by dietary changes. In particular, diets containing considerable amounts of vegetables (> 400 g/day) may reduce the relative risk of CRC by 40% (range, 30–50%) (van't Veer *et al*., 2000).

There are two different ways by which dietary intake can contribute to human CRC. The first is the presence of genotoxic agents in food that are responsible for the initiation of CRC development. Diet unquestionably represents the main exogenous modifier in the aetiology of CRC. For example, the two exogenous factors HAs and PAHs that are predominantly ingested as pyrolysis products present in food cooking at high temperature were shown to induce CRC in experimental *in vivo* studies (Hakura *et al*., 1998; Sugimura *et al.,* 2004). Beside exogenous substances, the potentially carcinogenic N-nitroso compounds (NOCs), either formed in foods such as preserved foods and/or endogenously formed by ingestion of processed meat containing nitrite or nitrate (Bingham *et al*.,

1996; Loh *et al*., 2011), can also induced CRC. The second concern is the variety of food components that can enhance or suppress human carcinogenesis through indirect ways. Food components like high fat diet, heme or high protein diet are high caloric diet which could promote CRC; all these potential food components have been tested in experimental studies in rodents (Bastide *et al*., 2011; Santarelli *et al*., 2008). Typical examples are high-fat mixed lipids diet containing high levels of saturated fatty acids (such as those in Western diets) (Rao *et al*., 2001), a high intake of dietary heme (Sesink *et al*., 2000,2001) and the increased DNA damage due to high dietary protein such as cooked red meat or casein (Toden *et al*., 2006, 2007a).

Although there is epidemiological evidence for a strong link between diet and CRC risk, the mechanisms by which diet might protect against CRC are still unclear. Dietary proteins, especially in red and processed meats, have been implicated as a positive risk factor for CRC (Marques-Vidal *et al.,* 2006; Santarelli *et al*., 2008), while starch (resistant starch) and dietary fibers that are not digested in the small intestine appear to be protective (Park *et al.,* 2005). Even if it is unclear which dietary component can either induce or protect from cancer, macronutrients like fat, fibers and proteins are likely to play a major role. The main reason for nutrition-dependent diseases in affluent society, however, is over-nutrition and malnutrition characterized by high-energy uptake and an unbalanced diet such as Western style diet. Possible protective macronutrients and micronutrients from fruits and vegetables have been studied in epidemiology and toxicity studies. The most consistent findings on diet have provided strong evidence about the dietary constituents that have a potential to protect against CRC. Mechanisms by which dietary antimutagens could protect against mutation by inhibiting mutagen uptake, endogenous formation, blocking or competition and suppression (van Breda *et al.,* 2008; Dashwood, 2002; Ferguson *et al.,* 2004; Vitaglione *et al.,* 2004) were observed, and many of these mechanisms would be predicted to operate in several sites, rather than being specific to the colon (Xu *et al*., 1999). The period of exposure of diet in large bowel is relatively long while this process is completed, increasing the risk of potential damage by carcinogens and toxins. At the intestine level, chemical substances such as nutrients, bioactive compounds, or contaminants, can have complex, multiple and variable interactions (**Fig.1.8**).

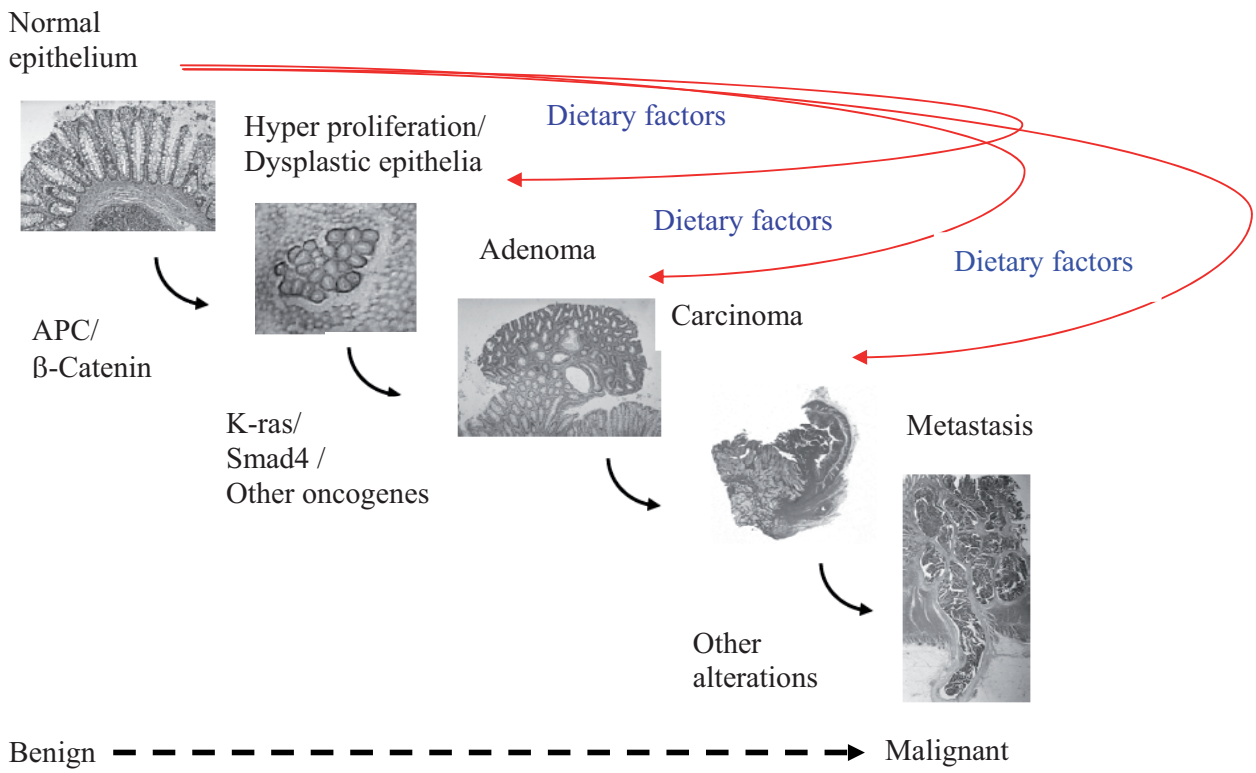


Figure 1.8. Intervention of dietary factors on difference stages of CRC.

3.1. Meat and meat carcinogens intake, and colorectal cancer: epidemiological studies

Correlation between CRC mortality and diet is remarkably strong. CRC is frequent in Western countries were red meat is frequently consumed; in contrast, this type of cancer is rare in less affluent countries where meat intake is low (Bingham *et al*., 2004). However, correlation is not causation, and it is clear that many other lifestyle factors are different in affluent and poor countries. The hypothesis that red meat and processed meat favours CRC must be tested. Numerous publications report a link between meat intake and CRC risk; most of them are retrospective case-control studies, some of them being prospective cohort studies in different countries worldwide.

Meat consumption has been reported a "convincing" risk factor for CRC in a large review by the World Cancer Research Fund (WCRF) (WCRF, 2007). However, conclusions with regard to meat are inconsistent: some studies reported decreased CRC risk or no association with white meat (Flood *et al*., 2003; Huxley *et al*., 2009; Marques-Vidal *et al.,* 2006; Tiemersma *et al*., 2002; Willett *et al*., 1990), while other studies suggested an increased CRC risk for red meat and processed meat (Corpet, 2011; Alexander *et al*., 2010; Santarelli *et al*., 2008).

Table 1.1. Mean daily intake of total, read and processed meat (g/day) by men and women in several European countries.

Country	Total meat[a]		Red meat		Processed meat	
	Men	Women	Men	Women	Men	Women
France*	145.4	94.5	61.2	39.1	41.8	27.5
UK**	108.1	72.3	40	24.6	38.4	22.3
Greece***	167.9	106.6	63.9	37.5	30.9	19.9
Spain**	78.8	47.1	45.3	25.5	10	5.8
Germany**	154.6	84.3	52.2	28.6	83.2	40.9
Italy**	140.1	86.1	57.8	40.8	33.5	19.6
Denmark**	141.1	88.3	69.6	44.1	51.9	25.3
Netherlands**	155.6	92.7	63.8	41.1	72.4	37.9

*[a]: Total meat: pork, beef, veal, lamb/mutton, poultry, game, rabbit, horse, goat, offal and processed meat. *: source Lafay et al., 2009, **: source Linseisen et al., 2002, ***: source Cosgrove et al., 2005.*

Total meat can be broken down into red meat, white meat (including chicken, game and turkey) and processed meat including cured and smoked meats (ham, bacon, sausages, hamburgers, salami and tinned meat). Red meat refers to mammalian meat that appears red before cooking; this includes beef, lamb, veal, pork and buffalo. Red meat is long established as an important dietary source of protein and essential nutrients including iron, zinc and vitamin B12 (COMA, 1998; Linseisen *et al*., 2002). Recent international correlation studies reporting that its consumption may increase the risk of CRC have led to a negative perception of the role of red meat in health (Cross *et al.,* 2007; Ferguson, 2010). In Europe, meat consumption varies from country to country, but on the whole meat consumption seems to increase. In the UK and Ireland, average daily intakes of total meat are respectively 108 and 168 g for men, 72 and 107 g for women (Cosgrove *et al*., 2005; Linseisen *et al*., 2002). In France, the food consumption survey INCA2 (which is a national individual survey on food consumption performed in 2006-2007) showed that red meat and processed meat are consumed by 92 and 91.1% of the French population, respectively (Lafay, 2009). **Table 1.1** gathers the red meat and processed meat consumptions reported for several countries in Europe.

3.1.1. Meat intake and colorectal cancer in different countries

International studies showed that countries where people eat a lot of red meat and processed meat are also countries where the risk of CRC is high. Since 1990, many epidemiological studies (including case-control and cohorts' studies) dealing with dietary impact (especially red meat and processed meat intake) on CRC were conducted worldwide, especially in the western populations where CRC was more developed. On the whole, their results were inconsistent (Oba *et al*., 2006). About 60 cohort and case-control studies of meat intake and CRC have been recently reviewed (Alexander *et al*., 2010; Randi *et al*., 2010); among them, 24 studies (19 cohort and 5 case-control studies) were conducted in USA and published from 1990 till 2010, 9 studies were in Europe, 4 in Japan, 2 in China, 1 Australia, 1 in Argentina and 2 in Canada.

In studies conducted in the USA, red meat and processed meat consumption and a highest CRC risk were reported with a relative risk (RR) of 3.57 in men, and RR of 3 for women with rapid acetylators (Chan *et al*., 2005; Giovannucci *et al*., 1994; Wei *et al*., 2004; Willett *et al.,* 1990). In European studies, from a Dutch prospective study after 8.5 years of follow-up, red meat intake and increased CRC risk among men were highest (odd ration (OR)=2.7; 95% confidential interval (CI): 1.1-6.7, the increased CRC risk was alsmost 3 times more from highest vs. lowest intake) (Tiemersma *et al*., 2002). The European Prospective Investigation into Cancer and Nutrition study (the EPIC cohort) consisted of approximately half a million of men and women from ten European countries who were followed for cancer incidence. In an evaluation of meat consumption among participants in the EPIC cohort, Norat *et al*. reported a positive association (RR=1.35, 95% CI: 0.96-1.88) of CRC with intake of red and processed meat (highest versus lowest intake) (Norat *et al*., 2005). In the cohort of French Women of the National Education System (E3N), for quartile 4 versus quartile 1, an increased risk of colorectal adenoma was observed with 58% for the meat-eaters pattern. Dietary patterns that reflect a Western way of life are associated with a higher risk of colorectal tumors (Kesse *et al*., 2006). On the opposite, the Netherlands Cohort Study observed no significant associations between intake of total and red meat and CRC (Brink *et al*., 2005; Lüchtenborg *et al*., 2005). In Asia, among the individual studies conducted in Japan and China, associations between red meat intake and CRC were generally closer to the null value compared with associations from studies conducted in other geographic locations. In a Japanese study on almost 50 000 participants, dedicated to investigate meat consumption and CRC, associations between beef (1–2 servings per week vs. almost never) and colorectal, colon and rectal cancer were approximately 1.0 or below among men and women, after adjustment for several important factors (Sato *et al*., 2006). Results for pork were more variable; the RR for colon cancer was 1.46 while the RR for rectal cancer was 0.74, but these associations were

not statistically significant. Of interest, the RRs for beef and proximal and distal tumors were close to 1.0, and the RR for pork and proximal tumors was 1.05, but the RR for distal tumors was 1.90, albeit not significant.

Interestingly in these studies, associations varied considerably by tumor site. In most of the USA studies, positive associations were reported for colon cancer with RR more than 1.5 (Fraser, 1999; Giovannucci *et al*., 1994; Hsing *et al*., 1998; Wei *et al*., 2004; Willett *et al*., 1990; Wu *et al.,* 2004). The other studies in Australia, Finland, Canada, Sweden, Japan and one study in USA were reported for rectal cancer and distal colon cancer with relative risk more than 1.5 (Chao *et al*., 2005; English *et al.,* 2004; Jarvinen *et al*., 2001; Kabat *et al*., 2007; Larsson *et al*., 2005a; Sato *et al*., 2006). Similar patterns of associations of dietary red meat and processed meat intake and CRC were reported by gender (Cross *et al*., 2007; Gaard *et al*., 1996; Hsing *et al*., 1998; Wei *et al*., 2004). Some studies reported high associations of red and processed meat in men with RR more than 1.5 (Khan *et al.* 2004; Hsing *et al*., 1998; Oba *et al*., 2006; Tiemersma *et al*., 2002; Wu *et al*., 2004) and the others reported such associations in women (Gaard *et al*., 1996; Kesse *et al*., 2006; Kabat *et al*., 2007; Oba *et al*., 2006; Willett *et al*., 1990).

3.1.2. Dose-response of meat intake and colorectal cancer

Most of the epidemiologic studies showed an increased risk to develop the CRC for those eating higher amounts of meat, associated closely with type of meat. Hence, dietary intake of red meat was associated with risk of CRC with highest vs. lowest quintile levels of consumption or frequency consumption.

A dose–response relationship between meat intake and CRC was observed in many epidemiological studies (**Table 1.2**). In particular, since 2000 three meta-analyses also concluded on a dose-response relationship between red or processed meat consumption and CRC. Sandhu *et al*. made a meta-analysis gathering 13 cohort studies, selected from 17 studies, according to pre-established quality criteria;, the pooled relative risks (95% CI) were 1.14 (CI: 1.04-1.25) per 100 g/day for all meat, 1.17 (CI: 1.05-1.31) per 100 g/day for red meat, and 1.49 (CI: 1.22-1.81) per 25 g/day for processed meat (Sandhu *et al*., 2001). Norat's meta-analysis gathers 23 cohort and case-control studies, selected out of 48 studies by using pre-established quality criteria; the RRs of CRC were 1.35 for the quartile of people eating the highest amount of red meat (including processed meat) and 1.31 for processed meat, and the RRs estimated by log-linear dose-response analysis were 1.24 (CI: 1.08-1.41) for an

intake of 120 g/day of red meat and 1.36 (CI: 1.15–1.61) for 30 g/day of processed meat (Norat *et al*., 2002). Similar estimation was obtained in the meta-analysis made by Larsson *et al*., where 15 prospective studies on red meat and 14 prospective studies on processed meat consumption were identified; the estimated summary RRs were 1.28 (95% CI: 1.18-1.39) for an intake of 120 g/day of red meat and 1.09 (95% CI: 1.05-1.13) for an intake of 30 g/day of processed meat (Larsson *et al*., 2006).

Table 1.2. Red or processed meat intake and risk of colorectal cancer.

Food type	Cancer type	Intake (g/day)	RR (95% CI)	Reference
Red meat	Colorectal	100	1.29(1.04-1.6)	WCRF., 2007
Red meat	Colorectal	25	1.03 (0.97–1.09)	Sørensen *et al*.,
Processed meat	Colorectal	25	0.99 (0.86–1.13)	2008
All meat	Colorectal	100	1.21 (1.1-1.33)	Sandhu *et al*.,
Red meat	Colorectal	100	1.30 (1.13-1.49)	2001
Processed meat	Colorectal	25	1.49 (1.22–1.81)	
Red meat	Colorectal	120	1.24 (1.08-1.41)	Norat *et al*., 2002
Processed meat	Colorectal	30	1.36 (1.15-1.61)	
Red meat	Colorectal	120	1.28 (1.18–1.39)	Larsson *et al*.,
Red meat	Colon	120	1.24 (1.12–1.38)	2006
Red meat	Rectal	120	1.63 (1.24–2.14)	
Processed meat	Colorectal	30	1.09 (1.05–1.13)	
Processed meat	Colon	30	1.10 (1.05–1.16)	
Processed meat	Rectal	30	1.07 (0.98–1.18)	

As shown in **Table 1.3** and **Table 1.4**, the amount of red meat and processed meat intake is highly variable across studies. In particular, the cut-points of exposure categories differ considerably (e.g. >80 g/day vs. <10 g/day; >94/day vs. <52 g/day for red meat, 6.9–19.2 g/day, 19.3–294.2 g/day for processed meat, etc.) as do the types of exposure metrics (e.g. >2 servings per month vs. <2 servings per month; 3-4 servings per week vs. almost never; 4th quartile of intake vs. 1st quartile of intake [undefined]). Among the epidemiological studies that have taken into account cooking methods (e.g. high-temperature cooking techniques: grilling, frying, barbecuing, etc.) and doneness levels (such as well-done and very well-done) of red meat and poultry, some studies suggested a modest positive relationship between diets high in heavily brown red meats and CRC (Butler *et al*., 2003; Navarro *et al*., 2004; Nowell *et al*., 2002; Probst-Hensch *et al*., 1997; Sinha *et al*., 1999). However Marchand *et*

al. found an association only when the relevant bioactivation phenotypes were considered (Le Marchand *et al*., 2002).

Table 1.3. Meat, red meat and processed meat intake and risk of colorectal cancer.

Food type	Cancer type	Intake	RR (95% CI)	Reference
Red meat	Colorectal	≥80 g/day vs.<10	1.17 (0.92-1.49)	Norat *et al.,* 2005
Red meat	Colon	≥80 g/day vs.<10	1.20 (0.88-1.61)	
Red meat	Rectal	≥80 g/day vs.<10	1.13 (0.74-1.71)	
Red meat	Colon	≥ 5/week vs. 0	1.43 (1-2.05)	Wei *et al*., 2004
Red meat	Rectal	≥ 5/week vs. 0	0.9 (0.47-1.75)	
Red meat	Colon	≥29.3 g/day	1.2 (0.9-1.7)	Butler *et al*., 2005
Red meat	Colon	≥1/week vs. 0	1.41 (0.9-2.21)	Singh *et al*., 1998
Red meat (WD/VWD)	Colon	≥20.6 g/day	1.4 (1.0-2.0)	
Red meat (R/M)	Colorectal	12.3–141.6 g/day	1.12 (0.80–1.57)	Martinez *et al*., 2007
Red meat (WD/VWD)	Colorectal	14.5–151.7 g/day	1.33 (0.93–1.89)	
Red meat (R/M)	Colorectal	27.8-393.7 g/day	1.09 (0.86-1.39)	Sinha *et al*.,2005b
Red meat (WD)	Colorectal	20.1-510.7 g/day	1.02 (0.79-1.31)	
Red meat	Colorectal	≥28.5 g/day	1.3 (1.0–1.8)	Girard *et al*., 2008
Red meat (WD/VWD)	Colon	≥21.5 g/day	1.3 (1.0–1.7)	
Meat (WD)	Colorectal	>2 servings/week vs. ≤2	1.57 (1.27-1.93)	Cotterchio *et al*., 2008

R: rare, M: Medium, WD: Well done, VWD: very well done

Table 1.4. Red meat and processed meat intake (high vs. low) and risk of CRC.

Food type	Cancer type	Intake	RR (95% CI)	Reference
Red meat (WD/VWD)	Colorectal	Low quartile	1.91 (0.85–4.41)	Nowell *et al.*, 2002
	Colorectal	Medium quartile	2.42 (1.11–5.47)	
	Colorectal	High quartile	4.36 (2.08–9.60)	
Red meat (WD)	Rectum	Highest vs. lowest	1.3 (1-1.9)	Murtaugh *et al.*, 2004
All meat (darkly browned)	Colorectal	Highest vs. lowest	4.57 (3.1-6.73)	Navarro *et al.*, 2004
Red meat (WD/VWD)	Colon	Highest vs. lowest	1.7 (1.2-2.5)	Butler *et al.*, 2003
Red meat	Colon	Highest vs. lowest	1 (0.6-1.5)	Le Marchand *et al.*, 2001
Red meat (WD)	Colon	Highest vs. lowest	1.1 (0.8-1.6)	

WD: well done, VWD: very well done

3.1.3. Carcinogens in diet and colorectal cancer

3.1.3.1. Heterocyclic amines formation, occurrence and exposure

Heterocyclic amines (HAs) were first discovered in cooked foods by Professor Sugimura and his collaborators more than 25 years ago (Sugimura *et al.*, 1977 and 2004). In 1983, Nagao *et al.* demonstrated that specific HAs produced by pyrolysis of meats cooked at high temperatures were highly mutagenic (Nagao *et al.*, 1983). Since this discovery, the association between meat consumption and CRC risk has been postulated to involve increased exposure to HAs due to cooking methods. Later, formation of the HAs has been investigated in model systems, and a requirement for sugars, amino acids, and creatinine (or creatine) has been established (Jagerstad *et al.*, 1991; Skog *et al.*, 1992). These naturally occurring compounds are all present in red meat and can react together during cooking in Maillard reactions, through which food acquires its characteristic flavors, odors, and appearance. By-products of this chemistry include the HAs (Jägerstad *et al.*, 1983). Over 20 HAs compounds have been identified in cooked meats, being divided in two groups. On 2 mM nitrite treatment, Group I HAs (such as 3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-9H-pyrido[2,3-b]indole (AαC), 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole (MeAαC), 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-2)) lose their mutagenicity through

conversion of amino to hydroxyl groups, while the amino group of Group II HAs (such as 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8- trimethylimidazo-[4,5-f]quinoxaline (DiMeIQx), 2-amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline (7,8-DiMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)) is not changed (Tsuda *et al*., 1985). For the formation of Group II HAs, creatine or creatinine in muscles serves as a precursor of imidazo moieties, as reported by Jägerstad (Jägerstad *et al*., 1983).

The occurrence and formation of HAs in foods primarily depends on the characteristics of the food, such as the type of the food and the presence of precursors, water, and lipids. Secondarily, it depends on the cooking modes where the temperature and time are considered to be the most important factor involved in their formation (Knize *et al*., 2005; Robbana-Barnat *et al*., 1996; Skog *et al*., 1998). These compounds form in meats heated at 150°C or higher temperatures. Heat catalyzed degradation products of amino acids, with sugars and creatine (a key precursor present in muscle-meats) and HAs formation have been characterized in model systems (Skog *et al*., 1998). This first Group of HAs, the glutamic acid and tryptophan pyrolysate mutagens, are formed in proteins or produced directly from pyrolysis of these two amino acids heated at high temperature (>250°C) (Matsumoto *et al*., 1981). However, the concentrations of Group I HAs are generally much lower than those of Group II HAs in cooked meat. Thus, three of these cooked food-derived HAs (namely MeIQx, DiMeIQx and PhIP) are found more frequently than the others; together they account for the majority of the genotoxic potential of fried beef (Felton *et al*., 1986; Murray *et al.,* 1988; 1993). These three compounds have been detected in beef, lamb, pork, chicken, and fish, especially when char grilled, fried, or roasted.

HAs are present in nanogram per gram quantities in cooked meat as illustrated in **Table 1.5**. Nevertheless, human exposure to HAs, although low, is chronic as meat is consumed daily over a lifetime. Thus, depending upon dietary preferences, an individual's daily exposure to HAs is likely to range from microgram quantities to essentially zero in the case of vegetarians. Significantly, the incidence of cancer is approximately 60% lower in vegetarians than in non-vegetarians (Thorogood *et al*., 1994).

Table 1.5. Occurrence of HAs in several cooked foods (ng/g).

Food Type	Cooking method	Doneness level	ET* (°C)	Tine (min)	PhIP	MeIQx	DiMeIQx	Ref***
Bacon	Fry	M	175	4	0.2	0.1	0.2	Skog *et al.*, 1995a
		WD	200	4	0.6	0.7	0.3	
		VWD	225	8	4.5	23.7	1.4	
Pork Chop	Fry	M	175	11	0.02	0.2	0.04	
		WD	200	9	0.02	0.2	0.05	
		VWD	225	8	4.8	2.6	1.1	
Beef, Ground	Fry	M	175	6	0.04	0.2	0.02	
		WD	200	6	0.5	1.2	0.4	
		VWD	225	5	1.1	2.2	0.8	
Beef Steak	Pan-fry	M	186	16	nd	1.9	nd	Sinha *et al.*, 1998a
		WD	189	26	6.5	4.1	nd	
		VWD	191	33	23.2	8.2	nd	
Beef Steak	Grill/Bar	M	260	16	4.7	0.6	nd	
		WD	260	24	7.3	0.8	nd	
		VWD	249	41	30	2.7	nd	
Chicken, Breast[a]	Pan-fry	M	197	14	12	1	1	Sinha *et al.*, 1995
		WD	202	28	37	2	2	
		VWD	211	36	70	3	4	
Chicken, Breast[b]	Grill/Br	M	180	10	27	nd	Nd	
		WD	177	40	140	2	1	
		VWD	260	30	480	9	2	
Chicken, Breast	Pan-fry	WD	190	62	25	nd	nd	
	Grill/Bar	WD	191	63	36	nd	nd	
Fish, Salmon	Pan-fry	M	67**	8	nd	nd	nd	Iwasaki *et al.*, 2010
		WD	83**	11.6	nd	nd	nd	
		VWD	102**	18.3	7.37	0.66	nd	
Fish, Cod	Pan-fry	M	175	10	0.05	nd	nd	Skog et al., 1997
		WD	200	10	0.4	nd	nd	
		VWD	225	10	2.2	0.9	nd	

: Surface temperature (External temperature); **: Internal temperature; *: Ref: reference [a]: chicken breast without skin; [b]: chicken breast with skin; nd: not detected; M : medium, WD: well-done, VWD: very well-done.*

The formation of these compounds in meats depends largely on cooking temperature, duration, cooking method and equipment (Arvidsson *et al*., 1997; Sinha *et al*., 1995; 1998). Many epidemiological studies show about dietary red meat intake such as red meat grill at well done, and dietary habit relate to increase in the etiology of human cancer (Sinha *et al*., 1999; Skog *et al*., 1998).

Recent studies have shown that estimated human dietary HAs intakes (categorized crudely, based on self reported preferences for meat type, cooking method and/or doneness) are associated with elevated risks of CRC. In the case of the US population, total HAs intakes estimated for children (up to 15 years) and adults (>30 years) were 11 and 7.0 ng/kg bw/day, respectively, with PhIP estimated to comprise approximately 65% of each intake. Pan-fried meats were the largest source of HAs in the diet, and chicken the largest source of HAs among the different meat types (Keating *et al.,* 2001)**.** Estimates of HAs daily intake for Spanish population was 606 ng/capita/day (Busquets *et al*., 2004); in Sweden, the total HAs daily intake ranged from none to 1816 ng/capita/day, with a mean intake of 160 ng/capita/day (Augustsson *et al*., 1997). In Asia, the average HAs daily intake in Malaysia has been reported recently to be 553.7 ng/capita/day; the intake of PhIP was the highest, followed by MeIQx and MeIQ (Jahurul *et al*., 2010). In Singapore, the estimated mean daily exposure to HAs was 49.95 ng/day; it was 50% higher among younger (20-39 years) compared with older individuals (Wong *et al.*, 2005).

3.1.3.2. Polycyclic aromatic hydrocarbons formation, occurrence and exposure

Polycyclic aromatic hydrocarbons **(**PAHs) are a group of chemicals composed of two or more fused aromatic rings made up of carbon and hydrogen; they are considered to be ubiquitous environmental contaminants. Generally, they have high melting and boiling points, low vapor pressure, and very low water solubility. For the general population, the major routes of exposure to PAHs are from food, tobacco smoking and environment. Food can be contaminated by environmental PAHs that are present in air (by deposition), soil (by transfer) or water (by deposition and transfer), or during processing and cooking. Indeed, processing of food (such as drying and smoking) and cooking of foods under high temperatures (grilling, roasting, frying, barbecuing) are major sources of PAHs contamination (Phillips, 1999). More than 100 types of PAHs are known in nature and at least 20 of them are measurable in most dietary items (Ramesh *et al*., 2004). The Scientific Committee on Food (SCF) of the European Commission concluded that 15 PAHs, show clear evidence of mutagenicity/genotoxicity in somatic cells based on *in vivo* experiments, 14 of these PAHs having also shown clear carcinogenic effects in various types of bioassays in experimental animals (SCF, 2002).

PAHs are formed by the incomplete combustion of organic matter (EFSA, 2008; WHO/IPCS, 1998); benzo[*a*]pyrene (BaP) has been the most commonly studied and measured PAHs, as it is considered to be the most toxic as well as a representative member of PAHs. Relative potencies of carcinogenic PAHs to BaP have been determined by comparison of data which come primarily from dermal studies, and BaP concentration is a good marker of carcinogenic PAHs levels in environmental samples (Butler *et al*., 1993). The order of potencies is consistent, and this scheme therefore can provide an indicator of PAHs potency relative to BaP, expressed as BaP equivalents. Controversy exists concerning the use of this expression of relative carcinogenicity, since not all PAHs induce cancer via identical mechanisms. Also, data on oral studies are scarce and absorption and metabolism may play an important role in the effects. Yet, SCF has concluded that BaP is a good indicator of the PAHs content (SCF, 2002). More recently, the European Food Safety Authority (EFSA) concluded that BaP is not a suitable marker for the occurrence of PAHs in food and that a system of four specific substances (benzo[*a*]pyrene, chrysene, benz[*a*]anthracene, benzo[*b*]fluoranthene) or eight specific substances (benzo[*a*]pyrene, chrysene, benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*g,h,i*]perylene, dibenz[*a,h*]anthracene and indeno[1,2,3-*c,d*]pyrene) would be the most suitable indicators of PAHs in food (EFSA, 2008).

The EFSA published a report of the data collection on PAHs in food. The mean content of BaP was 7.81 µg/kg in coffee and tea, 4.71 µg/kg in herb and species, 2.42 µg/kg in fats and oil, 2.21 µg/kg in fully preserved fish, 1.93 µg/kg in comminuted meat, 1.97 µg/kg in fresh molluscs, 0.19 µg/kg in fresh fish, 0.29 µg/kg in fresh meat, 0.76 µg/kg in processed meat, 0.41 µg/kg in fresh fruit, and 0.38 µg/kg in fresh vegetables (EFSA, 2008). Kazerouni *et al*. also analysed BaP in 200 food items in order to estimate its intake in an epidemiologic study. BaP in whole chicken roasted was 0.01 ng/g, in perch fillet pan-fried well-done 0.15 ng/g, in smoked fish 0.1 ng/g, in bacon pan-fried well-done 0.2 ng/g, in smoked ham 0.13 ng/g, in pork-chop pan fried well-done 0.01 ng/g, in hamburger pan-fried well-done 0.13 ng/g, in steak pan-fried well-done 0.12 ng/g, and in steak gilled/barbecued well-done 0.81 ng/g (Kazerouni *et al*., 2001).

The total daily intake of BaP due to the consumption of various food items investigated in a Korean population was estimated to be 124.55 ng/person/day, based on daily food consumption and the contaminant level of BaP (Lee *et al*., 2007). In Taiyuan (China), the median values of BaP equivalent daily exposure doses for children, adolescents, adults and seniors of male were estimated to be 392.42, 511.01, 571.56 and 532.56 ng/day, respectively, whereas those for the above population groups of female were found to be 355.16, 440.51, 487.64 and 444.85 ng/day, respectively (Xia *et al*., 2010). The mean estimated dietary intake of the sum of the 16 PAHs in Catalonia (Spain) in

2000 was as follows: male adults, 8.4 µg/day; adolescents, 8.2 µg/day; children, 7.4 µg/day; seniors, 6.3 µg/day; female adults, 6.3 µg/day (Falcó *et al*., 2003). A tendency to decrease of food exposure to PAHs was observed in this region in 2008, the estimated mean dietary intake for a standard male adult (70-kg body weight) being 6.72 µg/day, a lower value than those found in 2000 (8.42 µg/day), and 2006 surveys (12.04 µg/day) (Martí-Cid *et al*., 2008; Martorell *et al*., 2010).

3.1.3.3. HAs, PAHs and risk of CRC: epidemiological studies

Table 1.6 and **Table 1.7** show the main characteristics of several cohort and case-control studies on red meat, processed meat and meat mutagens intake and risk of CRC. A dose-response relation was statistically significant for several specific HAs (PhIP, MeIQx and DiMeIQx) and CRC, and has provided additional support for a positive association between CRC and HAs exposure (Butler *et al*., 2003; Murtaugh *et al*., 2004; Navarro *et al*., 2004; Nowell *et al*., 2002; Sinha *et al*., 2001). In addition to research on CRC, some epidemiological studies have evaluated cooking methods, meat doneness and surface browning (well-done/very well-done) and HAs exposure in relation to CRC and/or adenomatous polyps, precursors of CRC (Augusston *et al*., 1999; Gerhardsson de Verdier *et al*., 1991; Gunter *et al.,* 2005; Ishibe *et al*., 2002; Kampman *et al*., 1999; Probst-Hensch *et al,* 1997; Shin *et al*., 2007a; Shin *et al*., 2007b; Sinha *et al.*, 1997; Sinha *et al.,* 1999; Sinha *et al*., 2001; Sinha *et al*., 2005a ; Tiemersma *et al*., 2004; Wu *et al*., 2006). With the exception of two studies (Gunter *et al*, 2005; Tiemersma *et al*, 2004), many studies reported positive associations of colorectal polyps with intake of well-done meat and/or exposure to HAs.

Recent case-control studies suggest that PAHs may be better candidates than HAs to explain that overcooked meat is a CRC risk factor, but data on PAHs are insufficient to conclude. The study of Sinha *et al*. evaluated the dietary BaP intake and risk of colorectal adenoma; in cases, median BaP intake was 17 ng/person/day from meat and 76 ng/person/day from all foods. Intake of higher levels of BaP from meats led to a 2.8-fold increased risk when comparing the fifth to the first quintile. Increased risk of colorectal adenomas was more strongly associated with BaP intake estimated from all foods with RR 5.6 (2.2-14.2) of quartiles 5 vs. 1. This study provided evidence that dietary BaP may play a role in colorectal adenoma etiology (Sinha *et al*., 2005c).

Table 1.6. Colorectal cancer risk by Relative amount of meat-carcinogens in Cohort study.

Authors and country	Meat-carcinogens	Cancer type	RR	95% CI	Sexes	Age (years)
Rohrmann *et al*., 2007, Europe (EPIC)	PhIP	Colorectal ademona	1.47	1.13-1.93	Both	Unknown
	MeIQx	Colorectal ademona	1.27	0.97-1.68	Both	Unknown
	DiMeIQx	Colorectal ademona	1.18	0.92-1,53	Both	Unknown
Wu *et al*., 2006, USA	PhIP	Distal colon	1.11	0.85-1.46	Men	Unknown
	MeIQx	Distal colon	1.28	0.95-1.71	Men	Unknown
	DiMeIQx	Distal colon	1.08	0.86-1.37	Men	Unknown
Sinha *et al*., 2005b, USA	Total HAs	Colorectal cancer	1.03	0.77-1.39	Both	45-75
	DiMeIQx	Colorectal cancer	1.18	0.88-1.59	Both	45-75
	MeIQx	Colorectal cancer	1.09	0.81-1.47	Both	45-75
	PhIP	Colorectal cancer	1.03	0.77-1.39	Both	45-75
Cross *et al*., 2010, USA	PhIP	Colorectal cancer	1.03	0.77-1.39	Both	45-75
	PhIP	Colorectal cancer	0.99	0.87-1.12	Both	50-71
	MeIQx	Colorectal cancer	1.19	1.05-1.34	Both	50-71
	DiMeIQx	Colorectal cancer	1.17	1.05-1.29	Both	50-71

Cooking meat at high temperatures, and long periods of time with cooking methods that use direct or efficient transfer of heat such as frying or grilling, creates more potentially carcinogenic chemical by-products such as HAs or PAHs than do indirect heat methods such as stewing, steaming, and poaching (Cross *et al*., 2010; Santarelli *et al*., 2008). The cooking methods that give rise to the highest amounts of HAs and PAHs are pan-frying, barbecuing, and grilling of meats. High-temperature cooking of meat and fish has been related to colorectal adenoma (Probst-Hensch *et al*., 1997; Sinha *et al*., 1999, 2001) and CRC (Nowell *et al*., 2002). Fried meat with a heavily browned surface increased the risk of colon cancer by 2.8-fold and rectal cancer by 6-fold in a Swedish case-control study (559 cases and 505 controls) (Gerharddsson de Verdier *et al*., 1991).

The gene-diet interaction was replicated in a subsequent study, in which a 3-fold elevated risk was found among those who preferred well-done meat and had rapid CYP1A2 and NAT2 phenotypes (Le Marchand *et al*., 2001 and 2002). Furthermore, the association between well-done meats, HAs, mutagen index and CRC risk were found to be modified by NAT1, NAT2 and CYP1A2 genotypes (Kampman *et al*., 1999; Le Marchand *et al*., 2002). The associations among cooked meat-derived compound exposure and colon cancer are also found to be modified by the UGT1A7 genotype. For

example, a consumption equal to or greater than the median daily intake of DiMeIQx and having UGT1A7 low genotype was positively associated with colon cancer (OR, 2.4; 95% CI, 1.2-4.8), compared with a consumption lower than the median daily intake and UGT1A7 high/intermediate genotypes (Butler *et al*., 2005). Genetic susceptibility may modify the associations of some meat or meat preparation factors with the risk of colorectal cancer (Cotterchio *et al*., 2008; Murtaugh *et al*., 2005).

Table 1.7. Relative risk of meat-carcinogens in case-control studies.

Authors	Meat-carcinogens	Cancer type	RR	95% CI	Sexes	Age (years)
Butler *et al*., 2003	PhIP	Colon	0.9	0.6-1.5	Both	>40
	MeIQx	Colon	1.1	0.6-2	Both	>40
	DiMeIQx	Colon	1.8	1.1-3.1	Both	>40
	BaP	Colon	1.2	0.8-1.7	Both	>40
Gunter *et al*., 2005	PhIP	Total adenoma	1.01	0.58-1.73	Both	50-74
	MeIQ	Total adenoma	0.89	0.52-1.55	Both	50-74
	DiMeIQ	Total adenoma	1.15	0.69-1.91	Both	50-74
	BaP	Total adenoma	1.03	0.6-1.75	Both	50-74
Girard *et al*., 2008	PhIP	Colon	1.0	0.7-1.3	Both	40-84
	MeIQx	Colon	1.2	0.9-1.7	Both	40-84
	DiMeIQx	Colon	1.3	0.9-1.7	Both	40-84
	BaP	Colon	1.1	0.9-1.4	Both	40-84
Shin *et al*., 2008	PhIP	Colorectal polyp	1.39	1.11-1.74	Both	40-75
	MeIQx	Colorectal polyp	1.48	1.14-1.92	Both	40-75
	DiMeIQx	Colorectal polyp	1.37	1.1-1.71	Both	40-75
	BaP	Colorectal polyp	1.26	1.02-1.54	Both	40-75
Nowell *et al*., 2002	MeIQx	CRC	4.09	1.94-9.08	Both	20-88
Sinha *et al*., 2001	MeIQx	Colorectal adenomas	1.06	0.94-1.2	Both	46-71
	PhIP	Colorectal adenomas	1.00	0.98-1.03	Both	46-71

Meat consumption and CRC has been evaluated in hundreds of epidemiologic studies over the past three decades; however, the possible role of this food group on carcinogenesis is equivocal. In this comprehensive review, the currently available epidemiologic prospective studies of red meat and processed meat intake and CRC are summarized to provide a greater understanding of any potential

relationships. Collectively, associations between red meat consumption and CRC are generally weak in magnitude, with most relative risks below 1.50 and not statistically significant, and there is a lack of a clear dose–response trend. Results are variable by anatomic tumour site (colon vs. rectum) and by gender, as the epidemiologic data are not indicative of a positive association among women while most associations are weakly elevated among men.

4. Role of meat carcinogens in colorectal cancer development: *in vivo* studies

To our knowledge, rodents have been the preferred animal species for *in vivo* studies. We will first present in detail reported results for heterocyclic amines. Then, main results related to polycyclic aromatic hydrocarbons will be indicated.

4.1. Heterocyclic amines and CRC

Numerous experimental carcinogenicity studies of HAs have been carried. HAs are carcinogenic in rodents, and they induce tumors in multiple organs that include the oral cavity, liver, stomach, lung, colorectum, prostate and mammary glands, during long-term feeding studies (Sugimura *et al*., 2004). Examples of carcinogenic potential of HAs in multiple organs in rats and mice are presented in Annex I. It is noteworthy that some HAs can produce tumors in the colon, mammary glands, and prostate, which are common sites of neoplasms in Western countries and which incidence rates are increasing in Japan with westernization of dietary habits (Ito *et al.,* 1991; Shirai *et al*., 1997).

4.1.1. HAs induced CRC in rodents

The International Agency for Research on Cancer (IARC) collated the studies on carcinogenicity and classified IQ as probably carcinogenic to humans (Group 2A). MeIQ, MeIQx, PhIP and the apolar HAs (such as AαC, MeAαC, Glu-P-1, Glu-P-2, Trp-P-1, and Trp-P-2) were classified as possibly carcinogenic to humans (Group 2B) (IACR, 1993). HAs have several target organs such as colon, liver, prostate, mammary glands, and lymphoid tissues in both male and female rat (Ito *et al*., 1991). PhIP, the most abundant HAs formed in grilled beef, bacon, fish, and poultry (Knize *et al*., 1995; Skog *et al*., 1998), efficiently induces colon cancer in rats with highest incidence of adenocarcinomas (Imaida *et al*., 2001; Ito *et al*., 1991) and colon cancer in mice (Steffensen *et al*., 2001; Steffensen *et al*., 2006a,b). Macroscopic and histological features of some HA-induced tumors are shown in **Figure 1.9.**

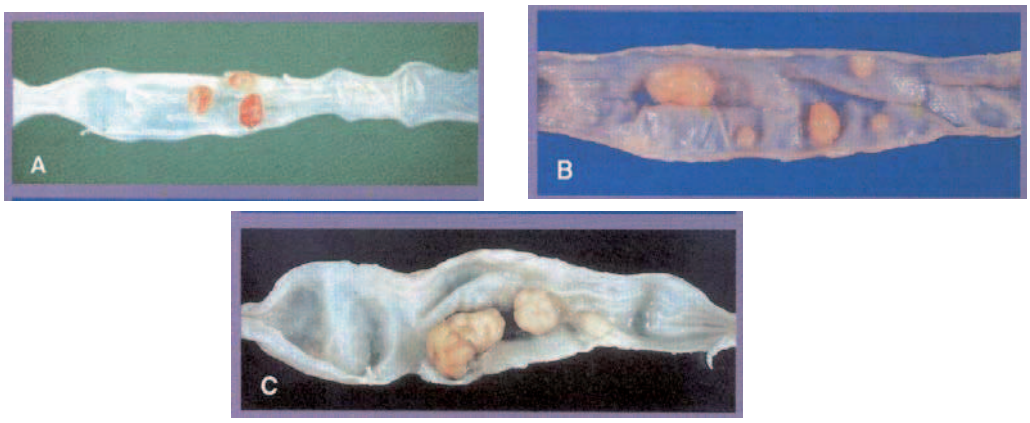


Figure 1.9. Macroscopic features of HA-induced cancers in experimental animals. Rat colon cancers induced by IQ (A), PhIP (B) and Glu-P-1 (C).

Tables 1.8 and **1.9** gather main experimental conditions and observed results of reported *in vivo* studies for HAs, in rats and mice respectively. In F344 rats fed a basal powdered diet containing 100 and 400 ppm PhIP, 43 and 55% of male rats developed carcinomas in the large intestine within 104 and 52 weeks, respectively (Hasegawa *et al.*, 1993; Ito *et al.*, 1991). However, no large intestinal tumors developed in male or female rats given 25 ppm PhIP within 2 years (Hasegawa *et al.*, 1993). In another study, male F344 rats developed colon tumors, mostly adenocarcinomas, at week 60 after repeating the three cycles of PhIP-feeding at dose 100 ppm or 300 ppm (in diet) (Imaida *et al.*, 2001). Nagase analbuminemic rats developed adenocarcinomas in intestine tumor within the 311 days experimental period (Ochiai *et al.*, 1991). With regard to other HAs, following a high fat diet for 32 weeks, MeIQ or IQ increased the number of colon tumors, either adenomas or adenocarcinomas. Numbers of induced colon tumors were found equivalent for PhIP and MeIQ, or a half for IQ (Ochiai *et al.*, 2005). IQ induced large intestine adenocarcinoma in rats (Takayama *et al.*, 1984a; Turesky *et al.*, 1996) in both sex, and more specifically in the proximal colon (Weisburger *et al.*, 1995). Colon cancers induced by PhIP occur preferentially in the middle or distal part of the colon, and feature mostly polypoid growth.

In the murine model for familial adenomatous popyposis (FAP), C57BL/6J-*Min*/+ (multiple intestinal neoplasia) mice are heterozygous for a nonsense APCMin (adenomatous polyposis coli) mutation, and therefore develop numerous spontaneous adenomas in the small intestine and colon. In pups directly exposed to PhIP *Min*/+ the number of colon tumors was significantly increased in both protocols of exposure (via breast milk and direct exposure) (Oshima *et al.*, 1995; Paulsen *et al.*, 1999; Steffensen *et al.*, 2001); besides, the increased numbers of colon tumors were dose-dependent

(Paulsen *et al.*, 1999). In mice fed 300 ppm of MeIQ in a basal diet for 92 weeks, a higher incidence of adenocarcinomas was reported in the colon and the caecum as compared to adenomas (Nagao *et al.*, 1998). After 20 weeks, in male mice fed with diet containing 300 ppm PhIP, MeIQx or IQ, the colon tumors (including adenocarcinomas) were found at a higher incidence in the PhIP-treated group, followed by the IQ- and MeIQx-treated groups (Nishikawa *et al.*, 2005). In CS7BL/6N female and male mice, AαC and MeIQx induced large intestinal tumors in a dose-dependent manner (Nishikawa *et al.*, 2005).

Table 1.8. HAs induced CRC in rat studies.

HAs	Dose in the diet	Test strain/ Sex	Study duration	CRC tumor incidence (%)	References
PhIP	25 or 100 ppm	F344 rats of both sexes	At 104 weeks	Colon adenocarcinomas: 43% males and 13% for females (100 ppm)	Hasegawa *et al.*, 1993
PhIP	100 or 300 ppm	Male F344 rats	At 60 weeks	Colon cancer 27% (300 ppm)	Imaida *et al.*, 2001
PhIP	400 ppm	F344 rats, both sexes	At 52 weeks	Colon carcinomas 55%	Ito *et al.*, 1991
PhIP	0.04, 0.03 and 0.01%	Male Nagase analbuminemic	At 311 days	Induced tumor in large intestine	Ochiai *et al.*, 1991
IQ	75 ppm	F344 rats, both sexes	At 15 or 18 months	Increased incidence of colon tumors	Weisburger *et al.*, 1995
IQ	300 ppm	F344 rats, both sexes	At 104 weeks	Induced colon tumors	Takayama *et al.*, 1984a
PhIP MeIQ IQ	400 ppm 300 ppm 300 ppm	Male F344 rats	At 32 weeks	Induced the ACF in the colon of rats and the number of colon tumors, either adenomas or adenocarcinomas (equivalent for PhIP and MeIQ or a half for IQ)	Ochiai *et al.*, 2005

Table 1.9. HAs induced CRC in mice studies.

HAs	Dose	Test strain/ Sex	Study duration	CRC tumor incidence (%)	References
MeIQ	300 ppm	Female BBM (C57BL/6N Tac (LIZ))	At 92 weeks	Incidence of adenocarcinomas in the colon was 42%, and 68% in the cecum	Nagao *et al.*, 1998
PhIP, IQ, MeIQx	300 ppm	Male C57BL/6J mice	At 20 weeks	Colon tumors: 22, 24, and 45% in the groups receiving MeIQx, IQ, and PhIP	Nishikawa *et al.*, 2005
PhIP	50 mg/kg b.w	F1 pups mice, both sexes	At 11 weeks of age	Increased the number of tumors slightly more in C57BL/6J mice than in A/J mice background but not in AKR/J background.	Steffensen *et al.*, 2006a
PhIP	1, 10 or 50 mg/kg b.w/day	C57BL/6J-(*Min*/+) mice	At 7 and 11 weeks after birth	Increased the incidences and numbers of tumors with a dose-dependent manner	Steffensen *et al.*, 2001
PhIP	25 or 50 mg/kg b.w	C57BL/6J-*Min*/+ pups and Apc^{Min}/Apc^{+} offspring	At 11 weeks of age	Increased the number of colon tumors: 3-to-4-fold via breast milk and 2-to-6 fold by direct exposure to 50 mg/kg b.w	Paulsen *et al.*, 1999
AαC	40.3 mg/kg b.w	Min/+ and +/+ offspring mice	At 11 weeks-old	AαC did affect intestinal tumorigenesis in *Min*/+ mice	Steffensen *et al.*, 2002
PhIP, IQ	50 mg/kg b.w	*Min*/+ and Apc^{Min}/Apc^{+} offspring	At 11 weeks-old	Induced colon cancer: PhIP (92% for male, 50% female), IQ (100% for male, 37.5% female)	Andreassen *et al.*, 2002

4.1.2. HAs induce aberrant crypt foci in rodent studies

As detailed previously in the chapter (§ 2.2), ACFs are putative preneoplastic lesions of the colon. They develop in rats and mice specifically after treatment with colon carcinogens; they do not appear after treatment with carcinogens that do not induce tumors in colon. PhIP, IQ, MeIQ, MeIQx and

Glu-P-1 were found to induce ACFs in various strains of rats and mice as summarized in **Tables 1.10** and **1.11**.

When rats were fed PhIP at concentrations of 25-400 ppm in the diet, they developed ACFs shortly after exposure to PhIP (Fujiwara *et al.*, 2003) in a dose dependent-manner. In other studies, F344 rats and ACI were fed with 400 ppm PhIP for three-cycle feeding; PhIP slightly induced ACF in rat colon (Ochiai *et al.*, 2003). The average number of ACFs induced was highest in BUF rats, lowest in ACI rats, and Wistar, F344 and BN strains were intermediate (Ishiguro *et al.*, 1999; Nakagama *et al.*, 2002). In pups and adult F344 rats exposed to PhIP, more ACFs were induced by PhIP in pup rats than in adults which indicated that the induction of PhIP in rats was age-dependent (Paulsen *et al.*, 2000). In rats, PhIP induced an increased number of ACFs through weeks 12 to 75, with a significant distribution from the distal to the proximal part of the large intestine between weeks 25 and 50; adenocarcinomas were found to develop at week 50 in the ascending colon and caecum (Tsukamoto *et al.*, 1999). Beside PhIP, other HAs (such as IQ, MeIQ, MeIQx, Glu-P-1) also induced ACFs in rat (Fujita *et al.,* 2002) and mice colon in a dose-related way (Tudek *et al.*, 1989). The number of ACFs observed 21 days after the initial IQ dose, was dose-related and crypts were found most frequently in the caecum (Tudek *et al.*, 1989). In female C57BLl6ByA mice, the number of ACFs induced by PhIP increased significantly over time (Hughes *et al.*, 2001), and small ACFs were predominant at all time-points (Kristiansen, 1996a; 1998; Sϕrensen *et al.*, 1996). In heterozygous Mlh1-deficient C57Bl/6 mice treated with PhIP, the increased frequency of ACFs was significantly greater in Mlh1^{-} /$^{-}$ than in wild-type littermates. This finding replies to the hypothesis that MMR-deficiency would increase the likelihood of PhIP-induced carcinogenic mutations (Ochiai *et al.*, 2002; Smith-Roe *et al.*, 2006b).

MeIQ induced aberrant crypts (ACs) in a dose-related way in rat and mice colon and produced more ACs (20 times more) in rats than in mice for the same dose (Tudek *et al.*, 1989). The ACF lesions induced by MeIQ might more preferentially be non-dysplastic (Ochiai *et al.*, 2005); this was detected even at week 6 and was observed from the earliest time point after treatment (Ochiai *et al.*, 2003). On the opposite, dysplastic lesions are induced by PhIP or IQ. In rodents, the number of dysplastic ACFs decreased as the number of colon tumors increased, indicating that, over time, the dysplastic ACF may develop into tumors (Steffensen *et al.*, 2001). The growth of ACFs can be expressed as crypt multiplicity ACs/ACFs (Paulsen *et al.*, 2000); it has been clearly demonstrated that the number of crypts/focus increases with time after the treatment. Dysplastic ACFs induced by PhIP in rats colon varied in size from 1 to 16 crypts.

Table 1.10. HAs induced ACFs in rat colon.

HAs	Dose	Treatment system	Study duration	Observations related to ACFs	References
PhIP	0.01, 0.1, 1, 10, 50, 100 or 400 ppm	Male F344 rats	16 weeks	Induced ACFs in colon from dose 50 ppm.	Fukushima *et al.*, 2004
PhIP	25, 100 or 400 ppm	Male F344 rats	8 weeks	Dose-dependent increasing ACs in the large intestine.	Fujiwara *et al.*, 2003
PhIP	400 ppm	Male F344 rats	14 weeks	Numbers of ACF was 6.6 ± 1.5 per rat in males and 1.9 ± 0.5 per rat in females.	Ochiai *et al.*, 1996
IQ PhIP	300 ppm 400 ppm	Male F344 rats	10 weeks	Increase of ACF numbers in rat colon treated with PhIP and IQ	Tsuda *et al.*, 1999 ; Kristiansen *et al.*, 1996b
PhIP	100 or 400 ppm	Male F344 and Wistar Kyoto	8 weeks	ACF: both rapid (F344) and slow-acetylator (wistar) rats with dose-dependant manner.	Purewal *et al.*, 2000
PhIP, Glu-P-1	0.05%	Male F344 rats	2, 4 and at 4, 8, 12, 16 weeks	Increased the number of ACFs in long feeding, and increased ACs in a preneoplastic lesion	Takahashi *et al.*, 1991
MeIQx	200 ppm	Male F344 rats	8 weeks	Induced ACF. GST-P + cell foci and colon ACF and ACs	Fujita *et al.*, 2002
MeIQx	100, 10, 1, 0.1, 0.01 0.001 ppm	Male F344 rats	16 weeks	Significantly increased of ACFs in the 100 ppm treated group	Tanakamaru *et al.*, 2001
PhIP, IQ, MeIQx	50 ppm	Male F344 rats	16 weeks	Numbers of colonic ACF were greater in the IQ+MeIQx group	Tsuda *et al.*, 1999
PhIP	400 ppm	F344, ACI, F1: (F344 xACI; ACI x F344), (F344xACI) F1v ACI	6 weeks	Induced ACFs in colon of F344, ACI, F1 and backcross rats	Nakagama *et al.*, 1999

Table 1.11. HAs induced ACFs in mice colon.

HAs	Dose	Treatment system	Study duration	Observations related to ACFs	References
AαC	500 or 800 ppm	CS7BL/6N mice, both sexes	At 14 weeks	ACF: 67% for males and 83% for females (500 ppm); 100% for both sexes at dose 800 ppm.	Okonogi *et al.*, 1997b
MeIQx	400 or 600 ppm			ACF: 40% for males and 60% for females (400 ppm); 100% for both sexes at dose 600 ppm.	
IQ	0.4, 2, 10 or 50 ppm	Male mice, (TSGp53 (+/-) heterozygous p53-deficient and wild type, C57BL/6)	At 32 weeks	ACFs were detected from 2 ppm to the highest treated groups. Observed dose-dependent increase of ACFs in transgenic mice groups.	Morimura *et al.*, 1999
IQ	300 ppm	Severe combined Immunodefici ency (SCID), both sexes	At 32, 52 and 78 weeks	Induced ACFs in colon of mice in all groups. Most ACFs were located in the proximal colon.	Hoshi *et al.*, 2004 ; Salim *et al.*, 1999
IQ	100 or 300 ppm		At 20 weeks	Induced incidence of ACF with time and dose dependence in both sexes.	
IQ	2, 10 or 50 ppm		At 23 and 30 weeks		

HAs induce ACFs in rats and mice, colon dyplastic ACFs having less than 4 crypts (Ochiai *et al.*, 2003). These lesions were more observed in pups than adult rats: 38% of ACFs had 5-17 crypts in rats exposed neonatally while no ACF with more than 5 crypts were seen in adult rats (Paulsen *et al.*, 2000). Dysplastic lesions contained crypts with homogeneously dense staining; in contrast non-dysplastic ACFs were composed of crypts with clear orifices, either split- or round-shaped (Ochiai *et al.*, 2005).

4.1.3. K-RAS genes and p53 alteration

A possible focus was done on mutation of the K-RAS gene, since it appears to be mutated early in the carcinogenesis process, and because HAs-DNA adduction can cause transition and transversion

mutation that may activate K-RAS genes (Jacoby *et al.*, 1992). However, it should be mentioned that although K-RAS, as well as p53 alterations, have been detected in rat colonic aberrant crypts treated with IQ, PhIP or Glu-p-1 (Kakiuchi *et al.*, 1993; Makino *et al.*, 1994; Tachino *et al.*, 1995), IQ also induced ACFs in p53-deficient mice; besides, wild type but no preneoplastic foci could be detected in neither transgenic or wild-type mice. These results suggest that germline p53 deficiency may slightly enhance the development of ACF in colons (Tachino *et al.*, 1995). Cell proliferation, p53 expression and c-K-RAS mutation are the most found in ACFs of the colon of rats given 100 ppm of MeIQx (Tanakamaru et al., 2001).

4.1.4. Wnt/APC/β-catenin signaling pathway- Alteration of the APC gene

The results from *in vivo* studies provide evidence for a major role of the β-catenin/APC pathway in the development of HAs-induced colon tumors, and give further weight to the view that regulation of β-catenin is critical to the tumor suppressive effects of APC during colon carcinogenesis (Dashwood *et al.*, 1998; Kakiuchi *et al.*, 1995; Tsukamoto *et al.*, 2000). The mutation spectra of β-catenin and ACP genes in colon lesions induced by HAs are presented in **Tables 1.12** and **1.13** for rats and mice respectively.

The mutation in either ß-catenin or APC gene is demonstrated by ß-catenin protein accumulation in the cytoplasm and nucleus. Mutations in APC, one G deletion occurring at G-stretch sequences 5'-GGGA-3' site to 5'-GGA-3' 3', and G to T transversion mutation in a G stretch induced by several HAs (such as PhIP, MeIQ, IQ and AαC) were observed in rat studies (Burnouf *et al.*, 2001; Kakiuchi *et al.*, 1995; Masumura *et al.*, 2000; Nagao *et al.*, 1997; Okonogi *et al.*, 1997b; Suzuki *et al.*, 1996; Zhang *et al.*, 1996). The predominating types of PhIP induced truncation mutations were found to be G to T transversion mutation in a G stretch (5'-tag-GGGGG-3' to 5'-tat GGGG-3') that occur at the boundary between intron 10 and exon 11, and G deletions occurring at 5'GGGA-3' sequences in exons 14 and 15 (Kakiuchi *et al.*, 1995). β-catenin mutations were observed at codons 32, 34, 36 or 38 in exon 2, the majority being G to T transversions (Ochiai *et al.*, 2003; Ubagai *et al.*, 2002). In the APC gene, 5'-GGGA-3' sites in exons 14 and 15 and a 5'-tagGGGGG-3' site at the junction of intron 10 and exon 11 have been reported to be mutation hot-spots (Ochiai *et al.*, 2003).
In the *lacI* of the Big Blue mouse treated with MeIQ, PhIP and AαC, G:C→T:A transversions were found, predominantly with MeIQ (Okonogi *et al.*, 1997a,b). Comparison of the *lacI* mutations in the rat colon with those identified in the mouse colon showed that the rate of G to T transversions was significantly ($p<0.05$) higher in the mouse (Okonogi *et al.*, 1997a).

Table 1.12. Mutation spectra of β-catenin and ACP gene in rat colon lesions induced by HAs.

HAs	Dose	Treatment system	Study duration	Mutation spectra	References
PhIP	400 ppm	Male F344 rats	At 1, 2, 4 and 6 weeks	Specific -1G mutations within 5'-GGGA-3' sequences of the APC	Burnouf *et al.*, 2001
PhIP	200 ppm	Big Blue F344 *lacI* transgenic, rat	At 68 days	Mutational spectra: G:C→T:A and G:C→C:G transversions and deletions of G:C base pair.	Stuart *et al.*, 2001
	400 ppm		At 67 days	Deletion of G in APC gene: 5'-GGGA-3' to 5'-GGA-3'	Okonogi *et al.*, 1997a
PhIP	400 ppm	Male F344 rats	> 43 weeks	Deletion of G in APC gene: 5'-GGGA-3' to 5'-GGA-3'.	Kakiuchi *et al.*, 1995
IQ	300 ppm			Missense mutation (T→C) at nucleotide 1567 and a nonsense mutation (C→T) at 2761.	Nagao *et al.*, 1997
IQ	20 mg/kg	Male big Blue F344 rats	At 2 weeks after the last treatment	G:C transversions. Detected a single G deletion in the sequence 5'-CGGGA-3'. Base substitution mutations at G in the sequence 5'-CGC/T-3' and for 1 bp deletions at the G doublet.	Bol *et al.*, 2000
IQ	130 mg/kg b.w	Male F344 rats	At 12 weeks after the last treatment	ACFs: GGT→GAT mutation in codon 12 and one contained a GGC→GCC mutation in codon 13.	Tachino *et al.*, 1995
MeIQx	100, 10, 1, 0.1, 0.01 or 0.001 ppm	Male F344 rats	At 16 weeks	GGT→GAT single based substitution in 100 ppm treated groups	Tanakamaru *et al.*, 2001

Table 1.13. Mutation spectra of β-catenin and ACP gene in mice colon lesions induced by HAs.

HAs	Dose	Treatment system	Study duration	Mutation spectra	References
PhIP	400 ppm	Homozygous *gpt* delta C57BL/6J transgenic mice	At 15 weeks	*gpt* mutations: single base pair substitutions and deletions at G:C base pairs. G:C→T:A transversions (81%). Spi (-) mutants: G:C base pair deletions (76%), which more than half occurred in monotonic G or C run sequences.	Masumura *et al.*, 2000
PhIP	100, 250 or 400 ppm	F1 (C57BL/6 x SWR) mice hemizygous	At 30, 60 or 90 days	Mutation spectral predominant: G:C →T:A transversions. Mutational hot spots for base-substitution mutations: at 5'GC-3' in runs of G and in 5'CGT-3' for these three HAs. PhIP- induced mutations: G:C base pair deletions preferentially within the sequence 5'-GGGA-3'	Suzuki *et al.*, 1996, Okonogi *et al.*, 1997b, Zhang *et al.*, 1996
AαC	800 ppm		At 30 or 45 days		
MeIQ	300 ppm	Female BBM (*lacI* transgenic) mice	At 1, 4 or 12 weeks		
PhIP	50 mg/kg b.w	*Mlh1*+/+ and *Mlh1*−/− mice	At 12 weeks after the last treatment	MF with G/C to T/A transversions with 69% in *Mlh1*+/+ and 23% in *Mlh1*−/− mouse colon. *IMlh1*−/− mice exhibited hypermutability to frameshifts, G/C to A/T transitions.	Smith-Roe *et al.*, 2006a
PhIP	10, 17.5 or 25 mg/kg	Min/+ pups mice	At 11 weeks after birth	PhIP-induced truncation mutations in the APC gene predominant: 60% G → T transversions, and 16% G deletion.	Paulsen *et al.*, 1999, Møllersen *et al.*, 2004, Masumura et al., 2000
PhIP	25, 50 mg/kg b.w				

Concerning *gpt* mutations from PhIP-treated C57BL/6J transgenic mice, 81% were single base pair substitutions and G:C→T:A transversions predominated; single base pair deletions at G:C base pairs were also observed (Møllersen *et al.*, 2004). Concerning Spi (-) mutants from PhIP-treated mice, 76% were G:C base pair deletions and more than half of these events occurred in monotonic G or C run sequences. G → T transversions (60%) and G deletions (16%) were found, indicating that these are the predominant types of PhIP-induced truncation mutations in the APC gene in *Min*/+ mice (Møllersen *et al.*, 2004; Paulsen *et al.*, 1999).

4.1.5. DNA-adducts

Formation of DNA-adducts is one of the mechanisms through which genotoxic agents may cause mutations and consequently induce cancer. Since the discovery of HAs in the late 1970s, a lot of work has been undergone to examine the capacity of these compounds to form DNA adducts. Numerous studies have demonstrated that HAs are potent mutagens in the Ames assay and induce tumors at multiple sites in laboratory animals; DNA-adducts have been frequently observed. MeIQ, IQ, MeIQx, PhIP, Trp-P-1, Trp-P-2, Glu-1, Glu-2, AαC and the other HAs are mutagenic toward *Salmonella typhimurium* (Nago *et al.,* 2000; Sugimura et al., 2004). It appears that MeIQ, IQ and 8-MeIQx are among the most potent mutagens ever tested in the Ames bacterial reversion assay (Sugimura *et al*., 2004). The mutagenic potency of HAs is dependent upon their chemical structure and their ability to undergo N-oxidation to form the reactive electrophilic aryl-nitrenium ion, which is able to bind covalently to DNA, therefore forming adducts (Hatch *et al.,* 2001; Schut *et al.*, 1999; Turesky, 2004). As an example, the pathway of PhIP metabolic activation is illustrated in **Figure 1.10**.

In vivo carcinogenicity studies related to HAs have been mainly carried out on rodents (mice and rats, see **Tables 1.14** and **1.15**). HAs were found to induce DNA-adducts in the colon of these animals. Guanine-adducts were detected in many *in vivo* studies, the major adduct being formed at the C8 position (Ikeda *et al.*, 2005, Møller *et al.*, 2002; Nerurkar *et al.*, 1995; Schut *et al.*, 1999). Two compounds (namely IQ and MeIQx) also form minor adducts at the N2 position of guanine (Turesky *et al.*, 1996, Schut *et al.*, 1999). A growing body of literature reported the mutation spectra induced by HAs-guanine adducts (Schut *et al*., 1999).

DNA-adducts were observed in the lower dose group (0.01 ppm) of rats treated with PhIP (Ochiai *et al.*, 2003; Turesky, 2004); induced-dG-C8-PhIP represented 35-45% of the total adducts (Ikeda *et al.*, 2005). In various rat strains treated with PhIP, the number of DNA-adducts in the colon was lower in

Gunn rats compared to Wistar rats (Malfatti *et al.*, 2005), and higher in F344 (rapid acetylator) rats than in Wistar Kyoto (slow acetylator) rats (Purewal *et al.*, 2000). PhIP DNA-adduct levels in colon mucosa of ACI rats were significantly higher than in F344 rats (Ishiguro *et al.*, 1999). DNA-adducts induced in the gastrointestinal tract by PhIP or *N*-hydroxy-PhIP were also observed in both rapid and slow acetylator congenic hamsters (Fretland *et al.*, 2001; Fretland *et al.*, 2003; Steffensen *et al.*, 2000). In the colon of big Blue Fischer male rats, IQ produced DNA-adducts with a dose-dependent relationship and oxidative DNA damage was detected by the endonuclease III enzyme and 7-hydro-8-oxo-2'-deoxyguanosine (Møller *et al.*, 2002).

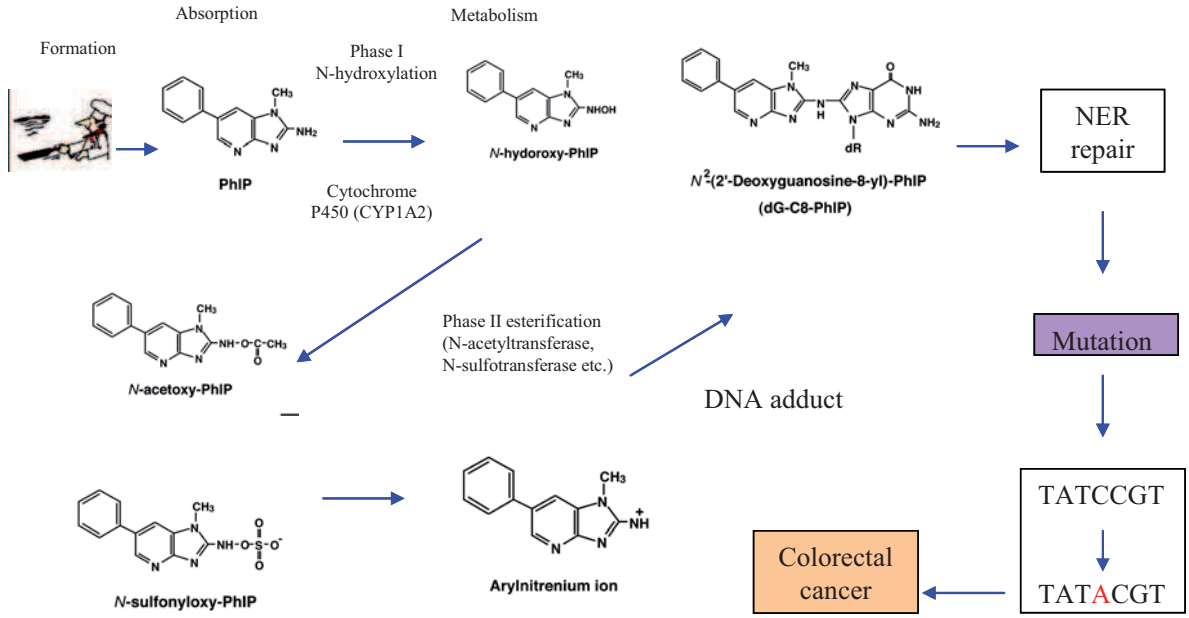


Figure 1.10. Pathway of metabolic activation of PhIP leading to DNA-adduct formation.

Table 1.14. DNA-adducts in colon of rats treated with HAs.

HAs	Dose	Treatment system	Reported DNA damages in colon	References
IQ + MeIQ + PhIP	250 mg/kg b.w	Male F344 rats	DNA damage with the 8-oxodGuo assay. Increased CYP450 1A1 and 1A2 in colon cell.	Tavan *et al.*, 2002
PhIP	100 µg/kg b.w	Male Wistar and Gunn rats	DNA-adducts in the colon of both strains with more adduct levels in the Wistar rats	Purewal *et al.*, 2000
PhIP	150 mg/kg b.w	Female rapid and slow acetylator rats congenic at the *Nat2 locus*	Major adducts were bound to the C8 position of deoxyguanosine. dG-C8-PhIP DNA-adducts were highest in colon with both starins.	Metry *et al.*, 2009
MeIQx	25 mg/kg b.w			
IQ	20, 70 or 200 mg/kg b.w	Male big Blue (Fischer) rats	DNA-adduct and DNA strand breaks in colon tissue.	Møller *et al.*, 2002
IQ	300 ppm	Male F344/Du Crj SPF rats	Oxidative DNA damage Increased 8-OHdG, acrolein-modified protein levels and BrdU-LI in colon (IQ+ $NaNO_2$).	Kitamura *et al.*, 2006
IQ	5, 25 or 50 mg/kg b.w	Young adult male F344 rats	DNA-adducts in colon.	Hoshi *et al.*, 2004
IQ	100 µmol/kg b.w	Male F344 rats	70% of the DNA-adducts occur in the colonic mucosa (pathway 1). Pathway 3 induced 30% of mucosal adducts with equal adduct levels in both layers.	Snyderwine *et al.*, 2002
MeIQ	0.03% or 80 mg/kg b.w	Male F344 rats	DNA damage in the large intestine	Bird *et al.*, 1989

b.w.: body weight

Table 1.15. DNA-adducts in the colon of mice (and hamsters) treated with HAs.

HAs	Dose	Treatment system	Reported DNA damages in colon	References
PhIP	100 mg/kg b.w	Male rapid and slow acetylator Syrian hamsters	DNA-adduct levels identified in colon and rectum	Fretland *et al.*, 2001
PhIP *N*-hydroxy-PhIP	100 mg/kg b.w 50 mg/kg b.w	Rapid and slow acetylator congenic Syrian hamsters	DNA-adduct levels in the gastrointestinal tract higher in *N*-hydroxy-PhIP treated group than in untreated group.	Fretland *et al.*, 2003
PhIP	340 mg/kg b.w	Male rapid and slow acetylator Bio.82.73/H*pat* hamsters	DNA-adducts were similar in the both strains. Detected single DNA-adducts as *N*-(DG--8- yl)-PhIP	Steffensen *et al.*, 2000
PhIP	50 mg/kg b.w	Male XPA-/- and XPA+/+ mice	More DNA-adduct formation in the colon; about twice level DNA-adducts in XPA-/- mice than in XPA+/+ mice.	Imaida *et al.*, 2000
Trp-P-2, IQ, MeIQ, MeIQx, PhIP	13 mg/kg b.w 40 mg/kg b.w	Male CD-1 mice	DNA damage in the colon for all HAs studied in Comet assay	Sasaki *et al.*, 1998
PhIP IQ	150 mg/kg b.w 25 or 75 mg/kg b.w	Female CYP1A2- null (knock-out, KO) and wild-type (WT) mice	Higher PhIP DNA-adduct levels in colon of WT mice than in KO mice. Higher IQ DNA-adduct levels in colon of WT mice than in KO mice only at the lowest dose.	Snyderwine *et al.*, 2002
IQ	0.01%	Male CDF mice	Increased DNA-adduct in colon	Herman *et al.*, 1997
IQ	20 mg/kg b.w	B6-Ah^{b}/Nat^{r}, B6A- Ah^{b}/Nat^{s}, B6-D- Ah^{d}/Nat^{s}, B6 AD Ah^{d}/Nat^{s} mice	Detected G adducts in colon, the predominant in N^{2}-(deoxyguano.sin-8-yl) -IQ. Ah-rapid acetylators had 3-fold more DNA-adducts than slow acetylators	Nerurkar *et al.*, 1995

4.1.6. Gene expression

Global gene expression analysis using a high-density oligonucleotide microarray revealed that 27 and 46 of 8749 genes or expressed sequence tags (EST) were over- and under-expressed, respectively, by three-fold or greater in PhIP-induced colon cancers (Fujiwara *et al.*, 2004). Differential gene expression profiles in colon epithelium of two rat strains (F344 and ACI rats) with distinct susceptibility to colon carcinogenesis after exposure to PhIP in combination with dietary high fat were studied; of 8799 entries on the RatU34A array, 74 genes were differentially expressed by three -fold or greater in normal regions of the colon epithelium between the susceptible F344 and resistant ACI strains (Fujiwara *et al.*, 2003). In the ACI strain, a mismatch repair gene, *Msh2*, was preferentially expressed, at approximately 20-fold as compared to the level in the F344 strain, along with a gene encoding a detoxification enzyme, catechol-O-methyltransferase. A total of 27 genes were over-expressed and 46 genes were under-expressed by three-fold or greater in common in colon cancers of both the F344 and ACI rats (Fujiwara *et al.*, 2003). In addition, the c-myc gene was expressed at a significantly higher level in cancerous than in normal tissues in both rat strains (Fujiwara *et al.*, 2003).

4.1.7. Aberrant differentiation of Paneth cells

Paneth cells (paneth cells are specialized secretory epithelial cells located at the bases of intestinal crypts) were observed within the lesions in adenomas and high grade dysplastic ACFs (Fujiwara *et al.*, 2004; Ishiguro *et al.*, 1999). A subset of genes whose expression is characteristic of Paneth cells, namely the intestinal-type defensin and matrilysin, were over-expressed in colon cancers. Hematoxylin, eosin and Alcian blue/periodic acid Schiff base (AB-PAS) staining revealed the presence of Paneth granules (Paneth cells of intestinal crypts contribute to host defense by producing antimicrobial peptides that are packaged as granules for secretion into the crypt lumen)in colon cancer cells, and lysozyme expression was also observed in cells with Paneth granules (Fujiwara *et al.*, 2004). Wnt/β-catenin signalling plays a key role in the homeostasis of the intestinal epithelium and colorectal cancer (Gregorieff *et al.*, 2005). However, whereas its role in the maintenance of the stem cell compartment has been clearly demonstrated, its role in the Paneth cell fate remains unclear. Yet, although molecular mechanisms underlying Paneth cell differentiation in colon cancer tissues have not been fully elucidated until now, activation of the Wnt/Apc/β-catenin signalling pathway could be a causative event. In rodent studies, activation of Wnt signalling has been reported to induce maturation of Paneth cells in intestinal crypts (van Es *et al.*, 2005; Fevr *et al.*, 2007). The appearance of Paneth cells may reflect the aberrant differentiation of colonic stem cells into cancer tissues. In mice study, Andreu *et al.* reported that acute activation of Wnt/β-catenin signalling induces *de novo*

specification of Paneth cells in both small intestine and colon, and that colon cancers resulting from APC mutations expressed many genes involved in Paneth cell differentiation; this suggests a key role for the Wnt/β-catenin pathway in Paneth cell differentiation (Andreu *et al.*, 2008).

4.1.8. Cell proliferation in CRC

The genetically altered epithelial cells have also a substantial impact on the development of CRC. In animal study, carcinogen-induced apoptosis in the target site of rat colonic epithelium could thus be a useful marker for the measurement of genotoxicity and/or initiating capability (Hirose *et al.*, 1996). In male F344 rats, the induction of apoptosis in the colonic epithelium after exposure to PhIP (100 mg/kg body weight) was detected (Hirose *et al.*, 1998). Cell proliferation was studied in colon of both *Min*/+ and +/+ mice treated with PhIP on day 12 or 36 after birth; proliferating cells were detected predominantly in the bottom half of the crypts (Steffensen *et al.*, 2005). IQ, MeIQ, Trp-P-1, Trp-P-2 and Glu-P-1 increased the incidence of nuclear aberrations per crypt on colon epithelial cells of C57BL/6J mice (Bird *et al.*, 1984). In F344 rats, IQ induced CRC by progressive inhibition of programmed cell death (apoptosis) via the deregulation of bloc-2 expression (Ilavaslii *et al.*, 1996).

4.1.9. Genomic instability: Microsatellites instability-Mismatch repair

Microsatellites instability (MI) was recently found to be associated with hereditary nonpolyposis-type CRC (Shibata *et al.*, 1994) and other sporadic and familial kinds of neoplasms (Han *et al.*, 1993; Honchel *et al.*, 1994). Disruption of the DNA mismatch repair (MMR) pathway results in elevated mutation rates, inappropriate survival of cells bearing DNA damage, and increased cancer risk. Indeed, DNA MMR contributes to genomic stability through the identification and correction of replication errors, thereby suppressing spontaneous mutations, and the activation of cell cycle arrest and apoptosis in response to DNA damage, thereby preventing the survival of damaged cells and reducing induced mutations (Kunkel *et al.*, 2005; Schofield *et al.*, 2003; Stojic *et al.*, 2004). Inactivation of MMR *via* epigenetic mechanisms, predominately hypermethylation of the MLH1 promoter, was associated with up to 15% of sporadic colorectal and other cancers (Duval *et al.*, 2002).

In male F344 rats, seven of eight colon tumors induced by PhIP showed alterations of at least one locus of microsatellite sequences; three PhIP-induced colon tumors had mutations in more than one microsatellite, their mutation rates being 2 of 85, 2 of 85, and 3 of 85 allele/microsatellite sequences, respectively (Canzian *et al.*, 1994). In *Mlh1*+/+ mouse colon, 69% of the PhIP-induced mutation frequency (MF) resulted from G/C to T/A transversions; in *Mlh1*−/− mouse colon, the reported predominance among PhIP-induced mutations of G/C to T/A transversions accounted for only 23%

of the total PhIP-induced MF. The induction of frameshifts, G/C to A/T and G/C to C/G base substitutions in *Mlh1*−/− colon was 11-, 10-, and 5-fold greater, respectively, than in *Mlh1*+/+ colon (Smith-Roe *et al.*, 2006b). Both the levels and types of mutation induced by PhIP can be influenced by the activity of the MMR system; MMR-deficiency would increase the likelihood of PhIP-induced carcinogenic mutations (Smith-Roe *et al.*, 2006b). In Msh2+/−/lacI and Msh2−/−/lacI double transgenic mice, PhIP induced a significant increase in mutation frequency in all genotypes of mice; this effect was more pronounced in Msh2−/− mice compared to Msh2+/+ and Msh2+/− mice (Smith-Roe *et al.*, 2006b). Msh2+/− mice displayed an increased level of G:C > T:A transversions and 71 frameshifts upon PhIP treatment. Loss of both Msh2 alleles mainly resulted in increased frequency of G:C > A:T transitions when exposed to PhIP (a defect in mismatch repair) (Zhang *et al.*, 2001).

4.2. Polycyclic aromatic hydrocarbons and CRC

As mentioned previously in this chapter, PAHs have been suspected of contributing to colon cancer. Indeed, several compounds of this chemical family were found to be carcinogenic in *in vivo* experiments, some of them being even mutagenic/genotoxic (SCF, 2002). Some examples are detailed below.

Absorption through the gastrointestinal tract occurs after ingestion of contaminated food and water. In experimental studies, both parent compounds and PAH metabolites were rapidly distributed throughout almost all the tissues, especially in lipid-rich tissues (IPCS, 1998). After entering the human body, PAHs undergo a series of biotransformation processes. Like many other carcinogens, PAHs are metabolized enzymatically to various metabolites, of which some are reactive. In the large group of enzymes involved in the carcinogenic process, cytochromes P450 CYP1A1, 1A2, 1B1 and 3A4 are the most important enzymes involved in the metabolism of PAHs (Pelkonen *et al.* 2003; Williams *et al*., 2000). Thus, during Phase I metabolism, PAHs are oxidized by cytochrome P450 enzymes to form highly reactive epoxide intermediates, which are then reduced or hydrolyzed by the epoxide hydroxylase enzyme to hydroxylated metabolites. However, PAHs can also be directly hydroxylated without the formation of the epoxide intermediate, as two other pathways were proposed for metabolic activation of PAHs: (i) the pathway *via* radical cation by one-electron oxidation; (iii) the ortho-quinone pathway by dihydrodiol dehydrogenase (Xue *et al*., 2005). In Phase II metabolism, the hydroxy-PAH metabolites are conjugated with glucuronic acid or sulfate to facilitate detoxification and excretion through urine or faeces (Grover, 1986; Pelkonen *et al*., 1982). Several metabolites can be identified from this process, including epoxides, DNA-adducts, dihydrodiols, and conjugated monohydroxy-PAHs (OH-PAHs). The major route of excretion of

PAHs (metabolites) involves accumulation in bile after conjugation with, for instance, glucuronic acid, and subsequent faecal excretion (Bond *et al*., 1986; Chipman *et al*., 1981). Generally, metabolites of small PAHs (with two to three rings) are excreted preferentially in the urine, mostly as glucuronic acid conjugates (Li *et al*., 2006), whereas metabolites of PAHs with higher molecular weight are excreted primarily in the faeces (Ramesh *et al*., 2004). The presence of unchanged PAHs in faeces has also been reported; in particular, BaP was found to be poorly absorbed, 88% of the ingested part of this compound being detected in faeces (Grova *et al*., 2002). In practice, the part of PAHs excreted unchanged in the faeces is known to depend on the bioavailability of the administered dose. Since a substantial part of PAHs is excreted in faeces, colon and rectum present a putative target for PAH-induced carcinogenicity. As a consequence, PAHs may be implicated in human colorectal carcinogenesis.

In recent years, in order to provide evidence about interactions of the genetic and the environmental factors, mutant mice with multiple intestinal neoplasias were widely used. Thus, APC^{Min} mice have been recently used to investigate the role of diet as a modifier of BaP metabolism and BaP induced colon tumors (Harris *et al.*, 2009). The BaP metabolite concentrations in plasma and colon of mice that received BaP through saturated fat were greater when compared to their control, mono- and poly-unsaturated counterparts (especially for reactive metabolites BaP-7,8-diol, BaP-3,6-dione and BaP-6,12-dione). Besides, an increased prevalence of adenomas was noticed in colon of mice that ingested BaP through saturated dietary fat compared to unsaturated fat and controls (P <0.05). These results therefore strongly suggest the involvement of BaP in colon carcinogenesis, as well as its potentiation by dietary fat (Harris *et al.*, 2009; Hakura *et al.*, 2000). Other oral studies in rats and mice gave evidence of BaP induced tumors in gastro-intestinal tract; hence, BaP high mutagenicity was reported in murine small intestine and colon (Hakura *et al*., 1998). In fact, it seems that DNA in human colon cells can be damaged by BaP, possibly derived from diet and/or tobacco smoke.

However, in order to exert their carcinogenic effects in colon or rectum, PAHs require metabolic activation. DNA-adduct formation in human colon epithelium proceeds *via* the diol epoxide pathway (Alexandrov *et al*., 1996). For instance, the pathway of B*a*P metabolism generates the ultimate carcinogen, BaP-7,8-diol-9,10-epoxide (BaPDE) which binds to DNA, causing bulky adducts. **Figure 1.11** presents the major metabolic pathway of BaP leading to this ultimate carcinogen.

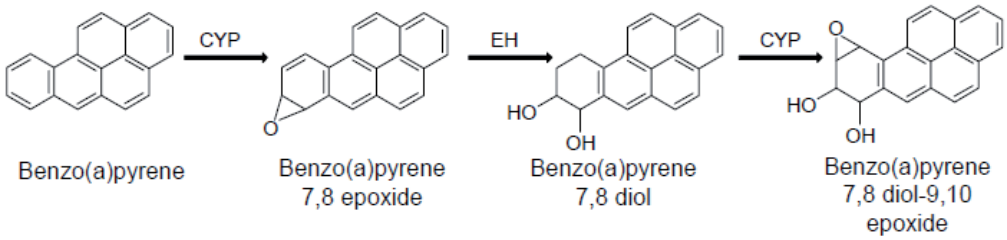


Figure 1.11. The major metabolic pathway of BaP leading to the ultimate carcinogen BaP-7,8-diol-9,10-epoxide (BaPDE). CYP: cytochrome P450; EH: epoxide hydrolase.

BaP-guanine adducts were the major adducts formed by both rat and human colonic DNA; however, BaP-adenine adducts were observed in rat colonic DNA but not in human colonic DNA (Autrup *et al*., 1980). Also, experimental studies revealed that cultured human colon cells can metabolize BaP by pathways similar to those found in human bronchus and in cells from experimental animals (Autrup *et al*., 1978).

The metabolic changes and susceptibility to BaP induced DNA-adduct formation, associated with adenoma formation, has been assessed in APCMin/+ mice; expression of CYP1A1/1A2, GSTa, m and p class xenobiotic metabolizing enzymes was assessed before and after BaP exposure, in macroscopic adenomas and uninvolved mucosa from APCMin/+ mice. Results showed greater CYP1A1/1A2 expression, responsiveness and greater genotoxic injury after BaP exposure in uninvolved mucosa than in adenomas (Sattar *et al.,* 1999).

5. Role of meat-based diet in colorectal cancer development: *in vivo* studies

Several *in vivo* studies have been undergone to investigate the effects of meat-based diet on CRC; their main results along with their experimental conditions are gathered in **Table 1.16**. Different mechanistic hypotheses derived from epidemiological and laboratory models could explain the possible involvement of red meat and processed meat in CRC risk. Four main mechanisms are proposed in the CRC risk associated with high intake of meats. Pro-cancer factors in red meat might be excess fat, excess proteins, excess iron, as well as exogenous and endogenous NOCs; in the case of processed meat, heat-induced mutagens could promote and/or initiate CRC according to rodents' studies.

5.1. Heme-based diet and CRC

In outbred Wistar rats fed a low-calcium diet, hemin-supplemented diet in non-initiated rats increased fat peroxidation and cytotoxic activity of faecal water and epithelial proliferation (Sawa *et al.*, 1998; Sesink *et al.*, 1999; 2000; 2001). These modifications were observed at all fat levels (Sesink *et al.*, 2000). Hemin-fed rats excreted much less host DNA in faeces than controls, which suggests that hemin may decrease cell differentiation and exfoliation of colonocytes in the gut lumen (Van Lieshout *et al.*, 2004). In azoxymethane (AOM)-initiated rats given a low-calcium diet, dietary hemin and hemoglobin promoted a dose-dependent growth of colon ACFs (Pierre *et al.*, 2003). Meat-based diets also promote ACFs and mucin depleted foci (MDF) in rats: MDF promotion by the high-heme blood sausage diet was greater than that induced by the medium-heme beef diet, while low-heme chicken diet did not promote MDF at all (Pierre *et al.*, 2004). The high-heme meat diets also increased the formation of lipoperoxides such as malondialdehyde in the gut lumen (Pierre *et al.*, 2004), as well as the excretion of a lipoperoxidation biomarker (1,4-dihydroxynonane mercapturic acid) in the urine of rats. The same biomarker was found in the urine of volunteers fed with blood sausage (high content of heme) (Pierre *et al.*, 2006). Subsequently, Pierre *et al.* challenged the hypothesis that nitrosyl heme in processed meat was more toxic than native heme in fresh meat (Pierre *et al.*, 2010). Cured meat can indeed promote colon carcinogenesis in rats. Dietary hemin, but not hemoglobin, could be used as a model agent to mimic the effects of processed meat in rats (Pierre *et al.*, 2010). In a recent study, Santarelli *et al*. demonstrated that the nitrosylation of heme was a key event in the promoting effect of processed meat in rats (Santarelli *et al.*, 2010).

5.2. High animal fat-based diet and CRC

Weanling male F344/N rats (six animals per group) were fed a Purina 5001 diet, half of them receiving the normal 4.5% fat feed and the other half receiving feed supplemented with 19% animal fat; after 26 weeks of treatment, the group of rats treated with tribromomethane (TBM) and fed a high fat diet showed a significant (near two-fold) increase in ACFs when compared to TBM exposed animals fed a normal diet (Geter *et al.*, 2004). Dietary proteins and fat from meat increased the incidence of colon tumors in F344 rats exposed (injection) to the carcinogen 1,2-dimethylhydrazine (DMH) (Reddy *et al.*, 1976). A 30% beef tallow diet given to rats after carcinogen injections increased the tumor occurrence, compared to the 5% fat diet fed controls; in contrast, this high fat diet had no effect when given simultaneously with the carcinogen (Bull *et al.*, 1979). A 20% fat diet also significantly increased the number of adenomas in the colon of DMH-initiated rats, whatever the protein and fat sources (meat, casein, corn oil or beef tallow) (Pence *et al.*, 1995). Diet containing

20% of mixed lipids (16% beef tallow, 10% lard, 12% butter fat, 30% hydrogenated soybean oil, 27% corn oil, and 5% peanut oil) induced a significantly increased incidence of colonic tumors and lower percentage of apoptotic colonic epithelial cells in rats. The mixed lipid diet caused significantly increased levels of cyclooxygenase 2 (COX-2) activities in colon tumors, and these tumors had enhanced levels of COX-2 expression. These observations demonstrate for the first time that mixed lipid diets containing high levels of saturated fatty acids (such as those in Western diets) promote colon carcinogenesis (Rao *et al.*, 2001). Rats fed a high fat diet *ad libitum* had more colon tumors compared to rats receiving a low fat diet (85 vs. 56% incidence, $p<0.05$). In 20% calorie-restricted pair-fed rats, the increased tumor incidence in rats receiving a high fat diet also compared to low fat diet was no longer significant (56 vs. 41% incidence). This study suggested that both fat and calories are CRC promoting factors (Kumar *et al.*, 1990).

5.3. High dietary protein content in diet and CRC

Diets that are very rich in fat or in proteins, such as red meat and processed meat, usually promote colorectal carcinogenesis in rats (Corpet *et al.*, 2011; Santarelli *et al.*, 2008). However, the evidence of CRC promotion is much weaker for high-protein diets than for high-fat diets (Visek *et al.*, 1991), and epidemiological studies do not suggest that protein intake is a risk factor for humans (Santarelli *et al.*, 2008; Williams *et al.*, 2010).

Few studies addressed the consequences of the level in dietary proteins on colon carcinogenesis. High beef protein and high soybean protein diets significantly increased the incidence of DMH-induced tumors in F344 rats compared with medium-protein control diets (Reddy *et al.*, 1976). High levels of dietary animal proteins (such as casein, red and/or processed meat) increased colonic DNA damages and thinned the colonic mucus barrier in rats (Toden *et al.*, 2007a,b). As expected, comet tail moment was greater and the mucus barrier thinner in rats fed 25% casein diet, and dietary protein induced colonocyte DNA damages in rats (Toden *et al.*, 2007c). In another study, rats were fed diets containing low (15%) and high (25%) levels of animal protein (casein) for 4 weeks; colonocyte genetic damage was measured by the Comet assay and was found two-fold higher in rats fed 25% protein as compared to those fed 15% protein (Bajka *et al.*, 2008).

Several mechanisms might explain CRC promotion by high protein diets. First, when dietary carcinogens enter the body as a part of the food matrix, to exert their toxicity they must pass the gastro-intestinal epithelium; as a consequence, the food matrix may have a major influence on the bioavailability of dietary carcinogens. In particular, the protein components of the food matrix can

influence the fate of dietary carcinogens in several ways. First, the carcinogens can be covalently bound to the proteins, thereby making the carcinogens non bioavailable and thus harmless. A second possibility is a non-covalent binding between the proteins and the carcinogens; the proteins can then act as transporters for the carcinogens, carrying them along the gastro-intestinal tract. In that case, once the proteins are digested, the carcinogens are released (either in the stomach or the small intestine). When the protein-carcinogen bond remains intact all along the small intestine, the protein-carcinogen complex will enter the colon; there, the protein is most likely fermented and the carcinogen will become available (Yoshida *et al*., 1991; Yoshida *et al*., 1992).

Secondly, a high protein diet or digestion resistant proteins lead to more protein entering the colon and being fermented by the gut microflora (MacFarlane *et al.*, 1991). Products of colonic protein degradation and metabolism include ammonia, phenols, indols and amines (**Fig. 1.12**) which have been shown to have some toxic effects *in vitro* and in animal models. These compounds are present in faecal samples, suggesting that they may exert some effects on the gut mucosa.

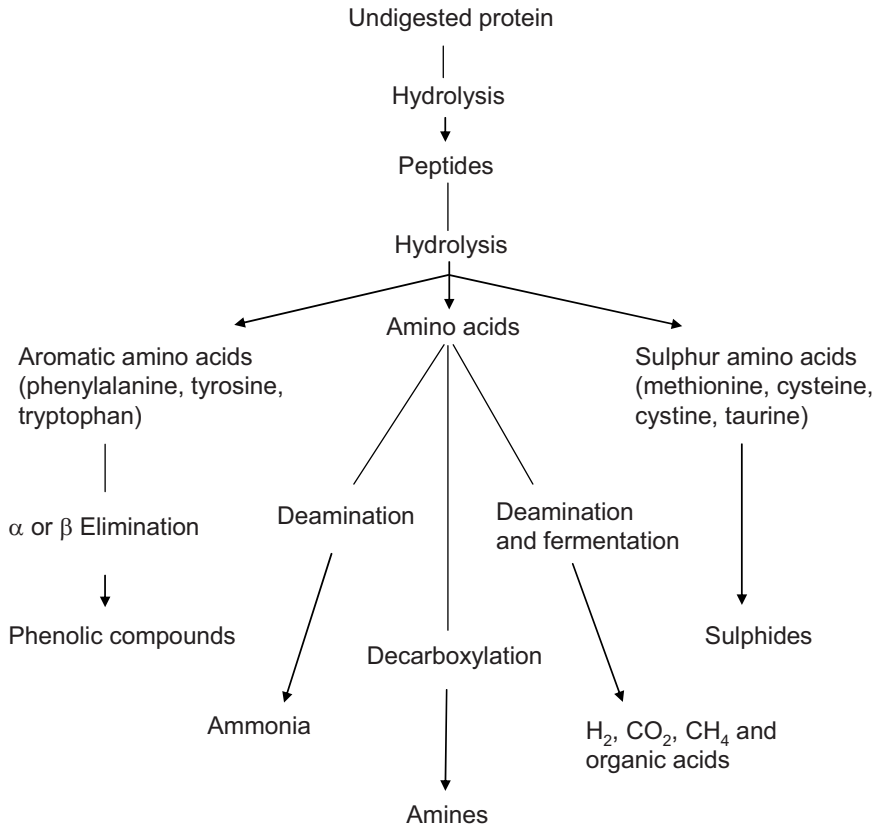


Figure 1.12. Metabolism of dietary proteins in the colon.

Human studies have shown that the colonic protein metabolism *via* the gut microflora is responsive to dietary proteins, as faecal ammonia and urinary phenolic compound concentrations increased in response upon high intake of protein-rich foods. Other toxic metabolites from dietary protein precursors such as N-nitroso compounds and sulphides can be also formed. Recent studies have shown that diets high in meat, fat and low in fibers increase human faecal water genotoxicity. It is possible that metabolites from colonic protein metabolism contribute to this increased genotoxicity during high meat intakes (Hughes *et al*., 2000).

Table 1.16 (Part ½). Studies on the effects of dietary meat, fat, heme and proteins on CRC in rodents.

Treatment system	Control diet (CD)	Experimental diet (ED)	Experimental procedure	Sacrified time	CRC potency/Endpoint	References
Female F344 rats	Low 5% fat and high fat diet with Ca (20 mmol/kg)	CD + 0.36 or 0.72 mmol/g hemoglobin + raw chicken, beef or blood sausage (BS). Beef + low Ca	7 days after the injection of AOM the rats were allowed free access to their ED diets for 100 days.	At 100 days	Heme in the form of hemoglobin in the Ca diet induced a strong dose-dependent increase in lipid peroxidation of faecal water indicated by thiobarbituric acid reactive substances. Heme in the form of meat induced specific sensitivity of normal cells, correlated to increased lipid peroxidation of faecal water. BS diet: diluted 5 times more the cytotoxicity assay, faecal water.	Pierre *et al.*, 2007
Female F344 rats	AIN-76 diet + $CaHPO_4$ (2.7 g/kg diet)	60% Chicken, beef or black pudding + hemoglobin 6.3 g/kg + $CaHPO_4$ (1.4, 1.6, 1.8 or 2.7 g/kg diet	7 days after the injection of AOM (20 mg/kg b.w) the rats were allowed free access to their ED for 100 days.	After 100 days	Diets with heme promoted the formation of MDF (greater in high-heme black pudding) and all meat diets promoted ACF formation. MDF promotion was correlated with high fecal water lipoperoxides and cytotoxicity.	Pierre *et al.*, 2004
Female F344 rats	Modified AIN-76 diet	Dietary beef meat (60% BM)	Rats were injected i.p. with the DMH and 7 days later, rats were allowed free access to their ED	At day 99 or 100	BM diet increased the number of ACFs and MDFs. Promotion was associated with increased faecal water thiobarbituric acid reactive substances (x4), cytotoxicity (x2), and urinary 1,4-dihydroxynonane mercapturic acid excretion (x15).	Pierre *et al.*, 2008

Table 1.16 (part 2/2). Studies on the effects of dietary meat, fat, heme and protein on CRC in rodents.

Treatment system	Control diet (CD)	Experimental diet (ED)	Experimental procedure	Sacrified time	CRC potency/Endpoint	References
Female F344 rats	AIN-76 diet: 5% safflower oil (SO) and 20 μmol Ca/g	Hemin (0.25, 0.5 and 1.5 μmol/g) or hemoglobin (1.5 and 3 μmol heme/g) diet	Rats were received a single i.p. injection of AOM (20 mg/kg). 7 days later, rats were fed with their ED.	After 97 or 99 days	Hemin diet: strikingly increased the ACF size and high hemin diet increased the number of ACFs/colon. Increased faecal water TBARs and cytotoxicity. Hemoglobin diets: increased the number of ACFs and faecal TBARs.	Pierre *et al.*, 2003
Female SD rats	Diet without fat	High fat diet (HFD), HF with beef (HFB), HF with cellulose (HFC) (22.4% fat)	Rats were fed with CD and ED for 3 weeks	At 3 weeks	HFD and HFB: reduced intestinal alkaline SMase and activities of acid and neutral SMases also (smaller than alkaline); and ceramidase activity and caspase-3 activity.	Yang *et al.*, 2002
Male Wistar rats	Standard diet	HLD (rich in ham) and VAHLD (rich in liver) diet	After 1 month of ED, rats were given one i.p. injection of DMH (15 mg/kg b.w.)	At 2 months after the starting exp. feeding	HLD: induced early down regulation of PPARc (–33.1%) and RARb (–53.1%) mRNA expression concomitant with an increase in levels of COX-2 and β-catenin.	Delage *et al.*, 2005
Male F344 rats		20% of mix lipid (MHML)	s.c. injections of AOM 15 mg/kg b.w. once weekly for 2 weeks and fed with ED till the end	At weeks 8, 23, and 38 after the last AOM	HFML: Lower % of apoptotic colonic epithelial cells and caused significantly increased levels of COX-2 activity in colon tumors.	Rao *et al.*, 2001

6. Influence of dietary nutrients on the prevention of colorectal cancer

The impact of dietary intake and life style risk factors on CRC risk has been extensively studied for a long time. The concept that different food components may initiate or prevent CRC was documented by several epidemiological studies and animal studies. The chemical and biological complexity of food, the difficulty in measuring habitual diets and the unavoidable changes in food constituents following a specific change in diet, contribute all to this complexity. As detailed previously, diet is clearly implicated in the occurrence of CRC, with risk factors for this disease including reduced consumption of vegetables, fiber, and starch, and conversely increased consumption of red meat and animal fat (Bruce *et al*., 2000). However, about 45% of all CRCs could be prevented, and diet plays a key role in modulating colon cancer risk (Birmingham *et al*., 2009; Gunter *et al*., 2006). Epidemiological and laboratory studies suggest that the consumption of fruits and vegetables is correlated with the decreased risk of colon cancer (Michels *et al*., 2006; van Breda *et al*., 2008). There are many plausible mechanisms by which intake of vegetables and fruits may prevent carcinogenesis. Firstly, plant foods contain a wide variety of anticancer phytochemicals with many potential bioactivities that may reduce cancer susceptibility; thus, many natural dietary compounds (such as antioxidants) have been isolated from fruits and vegetables and their health promoting properties have been demonstrated.
The majority of the effects found in the colon are changes in the expression of genes and proteins involved in apoptosis, cell cycle, cell proliferation and intracellular defense, in favor of reduced CRC risk. Furthermore, vegetables and vegetable components changed the expression of many more genes and proteins involved in other pathways for which biologic meaning is less clear (van Breda *et al*., 2008).

Indeed, diet may carry chemopreventive agents that could reduce the CRC risk. These agents can act according to two distinct ways: blocking mechanisms (inhibition of mutation and cancer initiation) and suppressing mechanisms (suppression of promotion, progression, invasion and metastasis) as illustrated in in **Figure 1.13**.

More than 300 agents have been tested in rodents: most of them were fed to rats, during or after the injection of a colon carcinogen. Fruit and vegetable intakes were not strongly associated with colon cancer risk overall, but may be associated with a lower risk of CRC (Marques-Vidal *et al.*, 2006). It has been hypothesized that the numerous constituents of vegetables and fruits, including micronutrients (nutritive compounds: e.g., carotenoids, vitamins C and E, folic acid and selenium),

dietary fibers and phytochemicals (non-nutritive bioactive compounds: e.g., flavonoids, indoles, isothiocyanates and glucosinolates), plus interactions among these constituents, might contribute to the ability of these foods to reduce cancer risk. These nutrients and bioactive compounds in fruits and vegetables can inhibit carcinogenesis during the initiation, promotion and progression stages; they have been shown to inhibit the deactivation of pro-carcinogens, induce detoxification pathways, affect the cell cycle by regulating cell cycle progression, influence cell to cell communication, quench free radicals, stimulate the immune system, modulate hormone metabolism, dilute and bind carcinogens, all of which should help to prevent the development of cancers (van Breda *et al*., 2008; Farombi, 2004; Murthy *et al*., 2009).

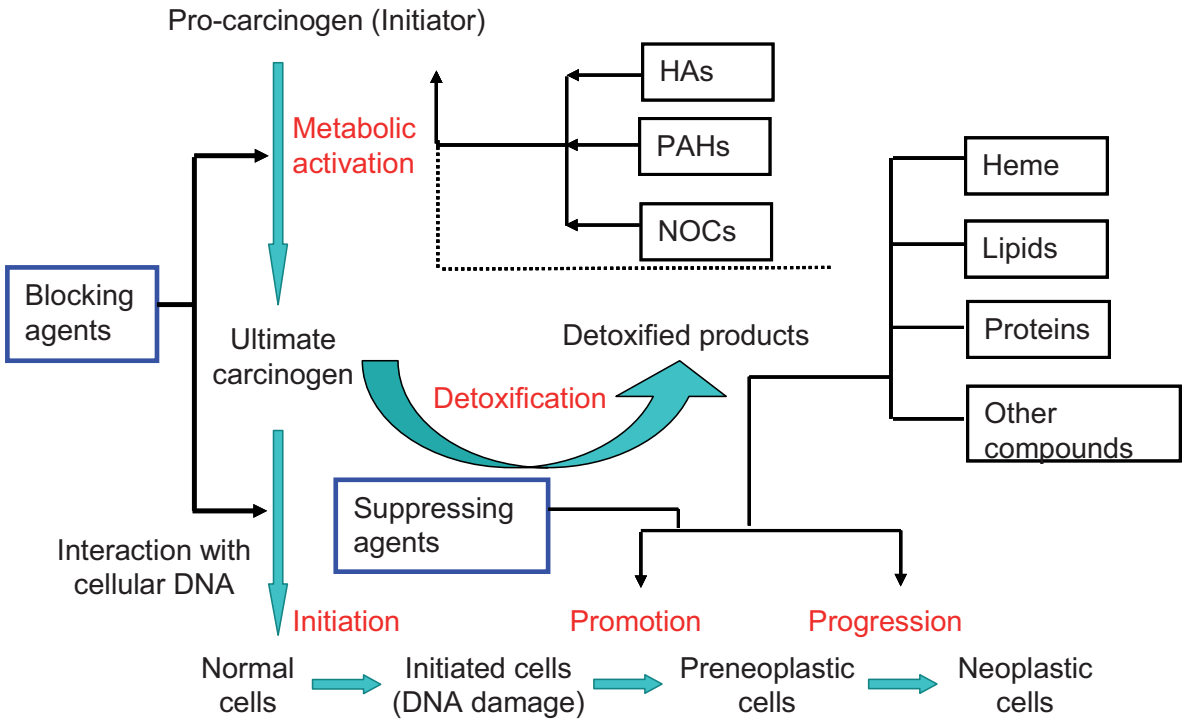


Figure 1.13. Scheme of initiation, promotion and progression of CRC with possible site interference of dietary components.

An analysis of dietary patterns or combinations of foods may provide insight regarding the influence of diet on the risk of CRC. We will detail below the possible CRC protective role of dietary fibers, as well the mechanisms involved in such effect. We also discuss how several microconstituents of the diet may be associated with reduced risk, including folate, methionine, calcium and vitamin D; short chain fatty acids also contribute to colonic health (Forte *et al*., 2008).

6.1. Dietary fibers and CRC

6.1.1. Dietary fibers in food items

Dietary fibers (DFs) are composed of plant cell walls and constitute a group of plant substances (cellulose, hemicellulose, lignin and other non-starch non polysaccharides) that are not digested in the human small intestine and can affect utilization of food by the body (Roberfroid *et al*., 2000; Stephen., 1994). The composition and properties of these plant cell walls vary according to cell type and plant species. Foods with dietary fibers include green and yellow vegetables, fruits and whole grains (Lottenberg *et al*., 2010). Cellulose, hemicellulose and pectin are carbohydrates, whereas lignin is a non-carbohydrate. DFs are more or less degradable by colon bacteria: 90-100% in the case of pectin, while this percentage is less for other fibers (50-80% for hemicellulose, 30-50% for cellulose); lignin is completely indigestible. Therefore, depending on the concentration of these components in the fiber, the digestibility and calorie value of fiber food vary. As physiological effects of dietary fibers partly depend on the extent of fermentation in the large intestine, it is influenced by chemical composition and physical form, such as solubility of the fiber (Stephen, 1994).

In practice, DFs can be divided into water-soluble and water-insoluble fractions. Water-soluble fibers in cereals are composed of non-starchy polysaccharides such as pectin, glucan, fructans and arabinoxylan; food sources rich in water-soluble dietary fiber components include legumes (beans, lentils), vegetables (such as Brussels sprouts and cabbage), fruits (such as apples and berries), oat bran and pysllium seeds (Manthey *et al.,* 1999). Water-soluble dietary fibers (SDFs), due to their higher degradability rate by the bacteria in the large intestine, have higher calorie content compared to water-insoluble dietary fibers. In addition, water-soluble dietary fibers can form viscous solutions; increased viscosity in the intestine slows down intestinal transit, delays gastric emptying (Anderson *et al*., 1986; Manthey *et al.,* 1999; Wisker *et al.,* 1985), and slows down glucose and sterol absorption by the intestine (Kahlon *et al.,* 1997; Wood *et al.,* 1990; Wood, 1991).

Water-insoluble dietary fibers (IDFs) include hemicellulose, cellulose and lignin; foodstuffs rich in IDFs are flaxseed, whole grain breakfast cereals, and vegetables such as celery and carrots. The rate and extent of fermentation of insoluble fibers in the colon are slower than those of soluble fibers. Vegetables and fruits (any plant product for that matter) contain both water soluble and insoluble fibers, but depending on the vegetable and fruit type or maturity, the soluble to insoluble fiber ratio may vary (Khanum *et al*., 2000). Several Asia fruits contain high content of diet fibers, as illustrated by the total dietary fiber (TDF) values of fruit guava (8.5%), jackfruit (3.5%, papaya (2.6%), mango (2.0%), banana (1.8%), peach (1.6%) and muskmelon (0.8%) (Ramulu *et al*., 2003). The insoluble, soluble, and total fiber contents (expressed in g/100 g of edible food portion) of

several foods are presented in Annexe I. Also, DFs contain minerals, antioxidants and other chemicals that are beneficial for health.

6.1.2. Possible mechanisms of dietary fibers protection against CRC

Studies examining the role of DFs as inhibitors of colon cancer in animal models have provided some insights into the potential mechanisms action for the prevention of CRC by DFs. They can be divided into two groups: (i) those where DFs are acting directly; (ii) those where this is the consequence of DFs being degraded by colonic bacterial enzymes and the resulting fermented products. Possible direct mechanisms include binding (adsorption) of carcinogens to non-degradable DFs, resulting in increased faecal bulk and shortened transit times; possible indirect mechanisms include lowering of the colon pH by the short-chain fatty acids produced by bacterial fermentation, and the specific effects of butyrate. However, besides these beneficial effects, there are also a number of possible mechanisms by which some DFs may enhance carcinogenesis; use of better-defined DFs in experimental studies will increase our understanding of the role of DFs in modulating CRC (Ferguson *et al*., 1996; Harris *et al*., 1993a).

- **Direct effects of dietary fibers**

Non-degraded or poorly degraded DFs can increase faecal bulk by their physical presence and by absorbing water; this increased faecal bulk results in shortened transit times through the gastrointestinal tract. Particulate DFs may also increase mobility through mechanical stimulation of multimodal mucosal receptors. Finally, non-degradable DFs may also protect by adsorbing carcinogens or promoters present in the digestive tract, and carrying them out of the body; thus, DFs can directly bind diverse colonic carcinogens (such as HAs and PAHs) and bile salts (secondary bile acids) (Ferguson *et al*., 1995; Harris *et al*., 1993b; Kestell *et al*., 1999; Lupton *et al*., 1999). In summary, DFs increase faecal bulk, dilute or adsorb carcinogens and bile salts, hasten transit, and therefore reduce contact time between carcinogens or promoters (e.g., secondary bile acids) and the luminal epithelium (Ferguson *et al*., 1996; Ferguson *et al*., 1998; Lipkin et al., 1999).

- **Indirect effects of dietary fibers**

Consumption of live bacteria (probiotics), non-digestible or limited digestible food constituents such as oligosaccharides (prebiotics) and polyphenols, or both (synbiotics), are recognized to modify the numbers and types of microbes, and have been reported to reduce colon cancer risk experimentally (Davis *et al*., 2009; Mai, 2004; Rastall, 2004). In particular, DFs can exert marked changes on the colonic microflora and the luminal environment (Topping *et al*., 2001) bringing

about changes in bacterial species and affecting production of certain microbial enzymes that are thought to be important in activation of carcinogens or co-carcinogens (Freeman, 1986); indeed, as detailed in Annexe I, the human microbiota has key physiological functions that might be related to cancer risk. Thus, DFs modified microbial composition in the gastrointestinal tract by increasing the bifidobacteria and decreasing the less desirable microbes, as well as causing changes in the levels of unidentified species (Apajalahti *et al*., 2002; Bouhnik *et al*., 1999; Ito *et al*., 1990; Silvi *et al*., 1999). This effect has been shown to be both dose-dependent and related to the initial level of bifidobacteria, with individuals with the lowest starting populations showing the greatest increase, also reflected in *in vitro* observations (Rycroft *et al*., 2001).

Microbial enzymes (including nitroreductases, azoreductases, hydrolases and β-glucuronidase) can convert inactive compounds to active metabolites, which may exert adverse effects. For example, β-glucoronidase hydrolyzes glucuronic acid conjugates of HAs, forming reactive metabolites, which can damage the colonic mucosal cells (Humblot *et al*., 2004). Faecal bile acid level is also an important parameter related to the development of CRC, and supplementation of DFs decreased faecal bile acid concentration (Suzuki *et al*., 1992). The modifying effect of DFs on secondary bile acids and bacterial enzymes (reduced metabolic activity) that may play a role in carcinogenesis depends on the type of fiber consumed.

The other mechanism by which water-soluble DFs may modulate carcinogenesis is *via* fermentation to short-chain fatty acids (SCFAs) in the colon. Indeed, the fermentation of fibers by colonic micro-organisms results in the production of SCFAs and a lower pH of large bowel contents (Topping *et al*., 2001), metabolic events known to be associated with increased epithelial cell growth and to stimulate colonic cell proliferation (Lupton *et al*., 1993*;* Zhang *et al*., 1994). The lower pH inhibits the conversion of primary bile acids to secondary bile acids. With regards to SCFAs, butyrate had the strongest correlation with indices of cell proliferation; considering that butyrate is not a common end-product from the fermentation of lactobacilli and bifidobacteria, its production would originate from the fermentation by other intestinal flora and it has been found that butyrate is produced in the colon at varying concentrations depending on the type of prebiotics (Liong *et al*., 2007, 2008). The reduction of colonic cell proliferation and induction of differentiation in colonic epithelial cells have now led to increased numbers of clinical trials of butyrate in the treatment of ulcerative colitis (Reddy *et al.,* 1997).

Many studies also indicate that some dietary patterns can stimulate insulin resistance or secretion, such as elevated consumption of high-energy diet and high-saturated fatty acid intake. DF intake could play an interesting role in the management of metabolic syndrome such as insulin resistance through different mechanisms related to their dietary sources, specific chemical structures and

physical properties, or fermentability in the gut. The different types of DFs have been reported to take part in the control of body weight, glucose and lipid homeostasis, insulin sensitivity and in the regulation of many inflammation markers involved in the pathogenesis of metabolic syndrome, and which are also considered to be among its features (Galisteo *et al*., 2008).

DFs may help prevent or correct the insulin resistance by two mechanisms. First, there is supporting evidence that a high intake of DFs is associated with enhanced insulin sensitivity, and that DFs participle to the regulation of energy intake (Burton-Freeman, 2000; Howarth *et al*., 2001). DFs decrease energy intake, lowering energy density regime (decrease subsequent hunger), and increase post meal satiety; they decrease the digestibility of fat and proteins by increasing faecal loss, particularly the water-soluble fibers. Second, the insulin and insulin-like growth factors can stimulate proliferation of colorectal cells. High intake of refined carbohydrates and markers of insulin resistance are associated with CRC. The positive associations of glycemic index and load with CRC suggest a detrimental role of refined carbohydrates in the etiology of the disease (Franceschi *et al*., 2001). The water-soluble fibers, by forming a gel in the stomach, delay gastric emptying and reduce the absorption rate of nutrients (such as glucose); the result is a flattening of the curve of postprandial glucose levels and decreased insulin levels (lowering the postprandial glycemic and insulinemic responses) (Jenkins *et al*., 2000; Mclntosh *et al*., 2001).

6.2. Micronutrients and CRC

Micronutrients may protect against CRC, especially dietary calcium, folate, beta-carotene and vitamin C; for that reason, a wide range of studies has been conducted on the potential effects of micronutrient intake in CRC prevention. In epidemiological studies, dietary intake of vitamins (C, E), folate and beta-carotene have been shown to protect against CRC risk (Roswall *et al*., 2010). High intakes of micronutrients commonly found in plant and other foods (in particular, β-carotene, vitamins C and E, calcium) exhibit independent associations consistent with 30–70% reductions in colon cancer risk (Satia-Abouta *et al*., 2003). A case-control in Australia, aimed to see the protective effects of dietary micronutrients on CRC, found that the diet containing the dietary micronutrients involved in DNA methylation (folate, methionine, vitamins B6 and B12) as well as some with antioxidant properties (selenium, vitamins C and E) may play a role in lowering colorectal cancer risk; besides this study revealed that such protection can be achieved by dietary means alone (Kune *et al*., 2006). In another study, the associations of dietary intake of calcium, fibers and vitamins with CRC risk in a population-based prospective cohort study conducted among Chinese women in Shanghai suggested that calcium may protect against CRC development even at a low consumption level, compared to Western populations (Shin *et al*., 2006a).

6.2.1. Calcium

Dietary calcium can reduce CRC risk in meat-eaters. Significant reductions in risk have been shown for the consumption of dietary calcium, milk and dairy products in general. Supplemental calcium in the diet or drinking water has been reported to decrease the colonic epithelial hyperproliferation induced by bile and fatty acids, enteric resection, a nutritional stress diet, and to suppress induction of the tumor-promotion enzyme ornithine decarboxylase. Calcium has also demonstrated an inhibitory effect on experimental colon carcinogenesis by inhibiting the cytolytic and hyperproliferative effects of dietary heme and the heme-induced effects (Sesink *et al*., 2001). In several rodent models, supplemental dietary calcium decreased colonic cell hyperproliferation as well as carcinogen-induced colonic tumors. In normal mice and in mice carrying a targeted APC gene mutation, increased colonic polypoid hyperplasia was associated with a Western-style diet containing low calcium and vitamin D levels (Lipkin *et al*., 1995). The main pathway used by extracellular calcium to exert its chemopreventive actions is through activation of a calcium-sensing receptor; this results in increased levels of intracellular calcium (**Annexe I**), which may suppress the growth of transformed colon cells by modulating PKC, p38MAPK and APC pathways (MacLeod *et al*., 2007).

Similarly, the physiologically most active molecular form of vitamin D (1,25-dihydroxyvitamin D3 or 1,25(OH)2D3) inhibits cell proliferation, and induces differentiation and apoptosis in colon and other cell types through the regulation of growth factor/ cytokine synthesis related to obesity (e.g., TGFβ, IL6, IL8 and Wnt signaling components) (Kaler *et al*., 2009; Lamprecht *et al*., 2003). In addition, 1,25(OH)2D3 and extracellular calcium act jointly through the calcium-sensing receptor (CaR) to regulate cellular proliferation, differentiation, and function. Thus, vitamin D and calcium insufficiency, which results in impaired antimitogenic, proapoptotic and prodifferentiating signaling from the vitamin D receptor (VDR) and the CaR, contribute to the pathogenesis of colon cancer (Peterlik *et al.,* 2009). Consequently, the supplementation of a Western-type diet with calcium and vitamin D decreased colon tumor incidence and multiplicity in an animal model of intestinal cancer (Newmark *et al*., 2009).

6.2.2. Folate

Folate is a water-soluble B vitamin found abundantly in fresh fruits and leafy green vegetables; it provides one-carbon groups in methylation of DNA (Van Guelpen *et al.,* 2006), and contributes to DNA synthesis and replication as well as epigenetic regulation of gene expression (Sanjoaquin *et al*., 2005**)**. Therefore, folate deficiency might impair these processes and cause chromosomal breaks, as well as deleterious alterations in gene expression. Epidemiological data strongly suggest an inverse relationship between dietary folate intake and incidence of CRC. Consequently, high

dietary intakes of folate and vitamin B_6 may reduce the CRC risk in women (Zhang *et al.*, 2006). However, more recently, randomised controlled trials investigating folic acid as a secondary preventive agent in colorectal neoplasia have shed further light on the relationship between folate and colorectal carcinogenesis, fostering data from animal models that indicate opposed effects dependent on the timing of exposure in relation to the development of neoplastic foci. A 'dual-modulator' role for folate in colorectal carcinogenesis has thus been proposed, in which moderate dietary increases initiated before the establishment of neoplastic foci have a protective influence, whereas excessive intake or increased intake once early lesions are established increases tumorigenesis. Functional polymorphic variant genes encoding key enzymes in the folate metabolic pathway add a further layer of complexity to the relationships between folate and CRC risk (Hubner *et al.,* 2009).

6.2.3. Beta-Carotene

Carotenoids are natural pigments primarily found in plants (fruits and vegetables). The predominant carotenoids in plasma are beta-carotene, lycopene, lutein and cryptoxanthin. Carotenoids, long recognized for their antioxidant properties, are of increasing interest in relation to cancer because of their effect on the regulation of cell growth, modulation of gene expression, and, possibly, immune response (Rock *et al.,* 1997). Epidemiological studies have indicated that higher intake of carotenoids is associated with reduced rates of chronic diseases such as certain types of heart disease, cataract and macular degeneration and certain cancers (such as colon cancer). The exact mechanisms by which the carotenoids alter these disorders are not known, but recent studies have made advances in this area. The major dietary sources of lutein in subjects with colon cancer and in control subjects were spinachs, broccolis, lettuces, tomatoes, oranges, carrots, celery, and greens; lutein intake was inversely associated with colon cancer in both men and women (Slattery *et al*., 2000*)*. Moreover, β-carotene supplementation reduced the rate of colon cell proliferation in patients with adenomatous polyps (Cahill *et al*., 1993). *In vitro* studies on the effects of beta-carotene on colon cancer cell lines were recently completed; beta-carotene was hypothesized to modulate cell redox status through both anti- and pro-oxidant effects. In the case of colon cancer, beta-carotene may enhance cell apoptosis (cell death) through pro-oxidant activities and therefore would reduce the number of tumor cells. The growth inhibition of human colon adenocarcinoma cell lines (COLO 320 HSR, LS-174, HT-29 and WiDr) by inducing cell cycle arrest in G_2/M phase and apoptosis was tested *in vitro*. At inhibitory concentrations β-carotene reduced the expression of cyclin A, a key regulator of G_2/M progression; neither p21 nor p27, two cyclin kinase inhibitors, were significantly modified by carotenoid treatment. With respect to apoptosis induction, decreased levels of the apoptosis blocking proteins Bcl-2 and Bcl-xL were also observed; on the other hand, no changes in

expression of the apoptosis promoter protein Bax were detected (Palozza *et al*., 2002). Beta-Carotene can modulate the COX-2 pathway, in particular, showing the evidence that the down regulation of COX-2 by carotenoids occurred under steady-state conditions as well as after growth factor stimulation; it was accompanied by growth inhibitory effects and by a decreased production of PGE2 (Palozza *et al*., 2005). Concomitantly, protection by β-carotene against colon cancer was shown in animal models (Alabaster *et al*., 1995) with a significant potential to inhibit ACFs. The putative preneoplastic lesions in the colon and COX-2 expression were slightly reduced in rats on beta-carotene supplementation (Choi *et al*., 2006). Inhibitory effects of natural carotenoids (such as lycopene, lutein, beta-carotene and palm carotenes, a mixture of beta-carotene, p-carotene and lycopene) on the development of colonic ACFs were found in rats treated with colorectal carcinogens (Narisawa *et al*., 1996). However, the chemopreventive activity of carotenoids such as β-carotene and lycopen against colon carcinogenesis depends on the dose level (Raju *et al.,* 2005). Hence, dietary β-carotene at the levels of 0.001 -0.01% in foods (low dose) was effective to inhibit colon cancer development and also ACF formation in rats and mice (Raju *et al.,* 2005; Temple *et al*., 1987). However, β-carotene at high levels (0.2% and 1% in foods) did not affect colon cancer development in rats (Colacchio *et al*., 1989; Jones *et al*., 1989; Raju *et al.,* 2005).

7. Exposure assessment to colorectal carcinogens

As detailed previously in this chapter, when considering the human diet, it should be recognized that foods contain both mutagens and components that decrease cancer risk (such as antioxidants). Thus, nutritionally related cancers ultimately develop from an imbalance ratio between carcinogens and anti-carcinogens. Among food mutagens, we will focus here on NOCs (formed during food preservation), as well as HAs and PAHs (formed during cooking). For example, the most important criteria in the context of HAs and human CRC are the strength and consistency of the association, the presence of a dose-response relationship, and the biological plausibility that HAs are human carcinogens. To investigate CRC risk posed by HAs to humans, accurate estimation of exposure is needed. However, there is a paucity of human data due to a lack of appropriate investigative tools.

An accurate assessment of dietary intake of such toxic compounds is difficult, mainly because they are not naturally present in foods, and they are not included in standard food composition tables. Accurate assessment of human exposure to these compounds requires food questionnaires that address cooking methods and reliable methods for the analysis of both such compounds (ex: HAs) in cooked foods and biomarkers of exposure (Skog *et al*., 2002). In several studies, the exposure assessment to HAs and PAHs was developed. The development of a database with HAs and PAHs concentrations in commonly consumed meat and food items cooked by various cooking methods,

temperatures and degrees of doneness (Sinha *et al*., 1997), is of importance to improve exposure assessment. The complex food matrix, the low amounts of HAs and PAHs present (ng/g), and the need for several isolation steps in the analytical process, make accurate quantification difficult. Besides, food composition (for example the concentrations and relative amounts of naturally occurring precursors, such as creatine, free amino acids and sugars in the case of HAs, as well as the presence of enhancing or inhibiting compounds) is well-known to greatly influence the formation of HAs and PAHs. Cooking temperature and time are other important factors that affect the formation yield of such compounds (Skog *et al*., 2002).

Anyway, human exposure to HAs and PAHs may be estimated using dietary assessment in combination with analytical data and/or food contaminants databases development on these substances level in various foods.

7.1. Diet questionnaires and databases for HAs and PAHs

The primary method for exposure assessment to colorectum carcinogens was the development of food questionnaires. The food frequency questionnaires (FFQs) with meat photographs linked to each database of interested substances are used in a variety of case-control and cohort studies of cancer etiology. Each FFQ must adapt to each situation and study cases; however, several common points are always found in the FFQs. The FFQ is made of questions about the consumption of specific studied meat (e.g. beef, pork, poultry) or fish items using a matrix format, and includes questions on the cooking methods (such as grilled, barbecued, fried), doneness levels (raw, medium, well-done and very well-done), preservation methods (such as canned, smoked), frequency of consumption and portion size (Kobayashi *et al*., 2002; Sinha *et al*., 1997; Sinha *et al*., 2005a).

Beside FFQs, 24-hour recall and 7-day food records were also used in the assessment of HAs and PAHs intake. The assignment of values for HAs and PHAs to foods in the FFQs, 24-hour recall and/or 7-day food records followed standards of practice on food composition data sources (Pennington, 2008; Pillow *et al*., 1999). Multiple reference databases were searched from published literature, reflecting HAs and PHAs values in foods and alcoholic beverages, and each reference database was searched using the different terms and combinations of the listed terms. To develop HAs and PAHs databases, food information needed are: name, cooking methods, preservation methods, cooking doneness, temperature, and time. Compound information was also required (type, quantity, value type, analytical method, and sampling method), as well as publication information (year, author, and country) (Jakszyn *et al*., 2004). **Figure 1.15** summarizes the methodology used to estimate intake of meat carcinogens.

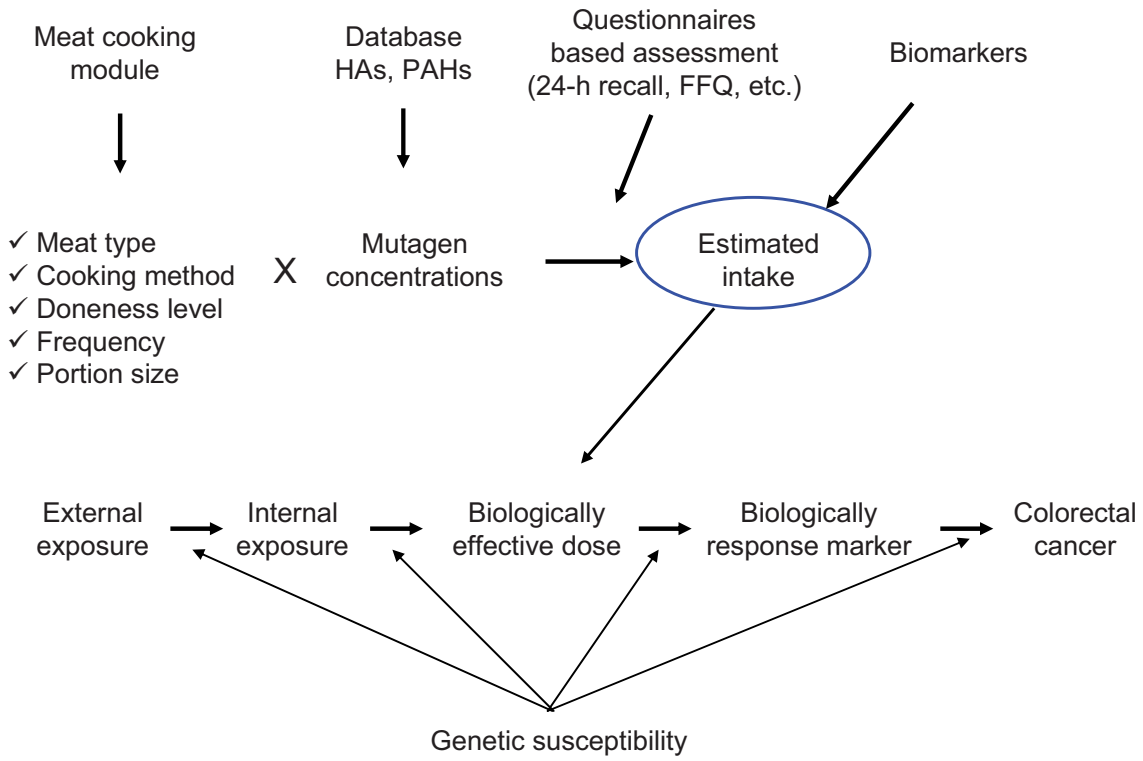


Figure 1.14. Method used to estimate intake of meat carcinogens and biomarkers (adapted from Sinha *et al*., 2005a).

Sinha *et al.* developed the first validated cooked meat module within a FFQ in the United States of America, and created databases to be used in conjunction with this FFQ to estimate intake of HAs and BaP (as a marker of PAHs) along with two other databases, one for total iron and heme iron in cooked meat and the other for nitrite, nitrate and NOCs in processed meats (Sinha *et al*., 2005a). One important point is to establish databases on HAs and PAHs in diet and cooked meat that are representative for the eating habits of the populations being studied.

By combining information on food consumption (using a semi-quantitative food frequency questionnaire including photos of fried meat) at an individual level with analytical data on HAs in various cooked food products, the estimated daily intake in Swedish population was found to range from 0 to almost 1816 ng/person/day (Augustsson *et al*., 1997). Still in Europe, of 21,462 subjects who participated in the European Prospective Investigation into Cancer and Nutrition (EPIC) in Heidelberg, the median total HAs intake from meat was 31 ng/person/day (mean: 69 ng/person/day) (Rohrmann *et al*., 2007). In China, the total HAs concentrations in foods ranged from <0.10 to 6.77 ng/g, of which Chinese-style roasted pork had the highest levels; the estimated mean daily exposure

to HAs was 49.95 ng/person/day; this was 50% higher among younger (20-39 years) compared with older individuals (Wong *et al*., 2005). In Japan, by using a self-reported food FFQ, the individual intake of six different HAs was estimated (Kobayashi *et al*., 2002).

Most individuals are exposed to PAHs predominantly from dietary sources (Fontcuberta *et al*., 2006); as mentioned before, PAHs are mainly formed during charcoal grilling, pan-frying and smoking of meat and fish. Vegetable oils and fats are also a significant source of PAHs in the diet, mostly related to the drying processes of the seeds where combustion gases may come into contact with the seeds (Speer *et al*., 1990).

Kazerouni *et al.* analyzed 200 food items for BaP and estimated its intake by the population in Washington (USA) metropolitan area from FFQ survey results. In this population, the bread/cereal/grain and grill/barbecued meat contributed 29 and 21%, respectively, to the mean daily intake of BaP (Kazerouni *et al*., 2001). Another interesting result from this study lies in the observation that, although for most people fish and seafood represent only a small part of the total diet, the contribution of this food group to the daily intake of PAHs in some individuals is still important. Mussels, clams and shrimps had the highest PAHs concentrations (22.4, 21.5, and 15.9 ng/g of fresh weight, respectively). From exposure to PAHs through consumption of edible marine species in Catalonia, Spain, the highest PAHs intake was found in women and girls (5.3 and 5.2 ng/kg b.w/day, respectively), female adolescents and female seniors having the lowest PAHs intakes (3.3 ng/kg b.w/day in both groups) (Llobet *et al.,* 2006). The assessment of PAHs intake by children and the general population in Estonia was also performed in another study;,the average intake of both BaP and the sum of 12 PAHs from meat products was estimated for children (age 1-16 years) on the basis of an individual food consumption questionnaire and, for the general population, based on national food consumption data. The highest total PAH concentrations detected were 16 µg/kg in smoked meat and ham, 19 µg/kg in smoked sausage and 6.5 µg/kg in smoked chicken samples; since smoking and grilling are prevalent meat-cooking methods in Estonia, the participation of meat products in the overall PAH intake was deemed to be significant (Reinik *et al*., 2007).

7.2. Internal exposure (biomarker) of meat-related mutagens/carcinogens

As presented just before, various methods of exposure assessment, such as questionnaires, sometimes combined with pictures of cooked meat, have been used in investigations on the relationship between meat-related mutagens/carcinogens (such as HAs, PAHs and NOCs) and CRC.

However, as the content of these compounds vary greatly with cooking conditions, it is difficult to obtain an accurate estimate of the exposure. To improve the exposure assessment, the use of biomarkers has also been investigated (**Fig. 1.16**).

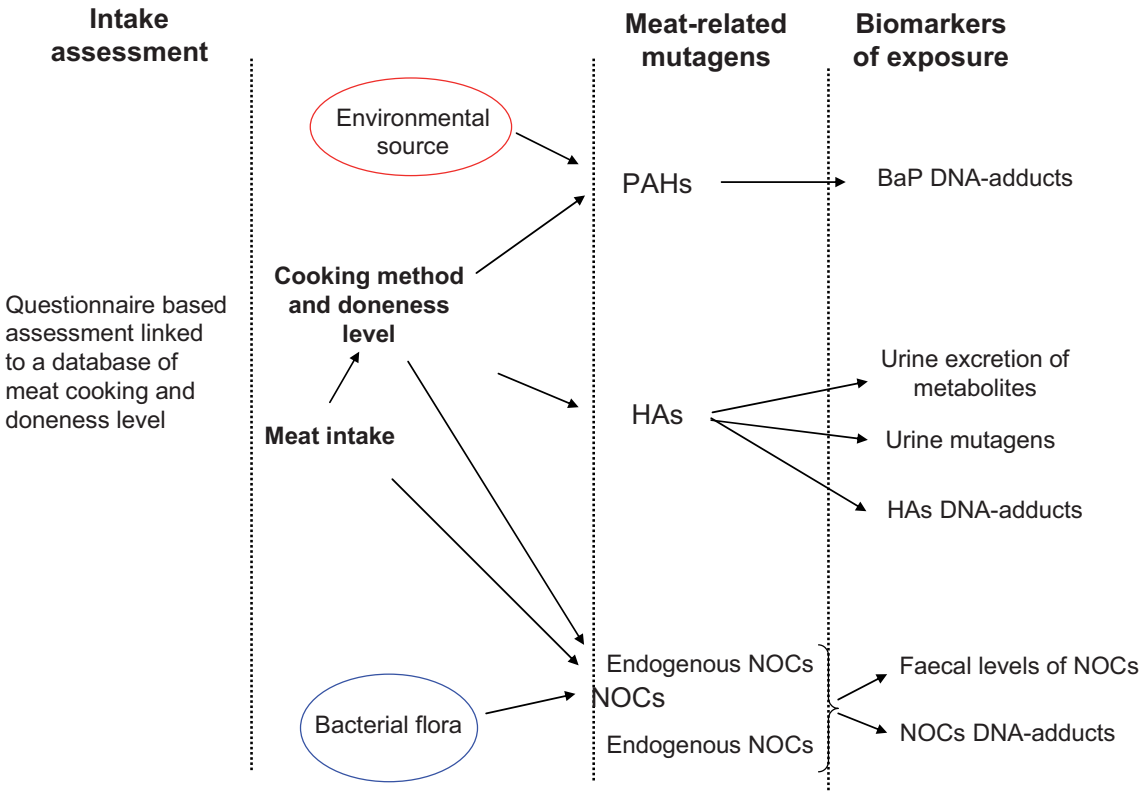


Figure 1.15. Some of the potential mechanisms linking meat and meat-related mutagens to biomarkers of colorectal cancer risk (adapted from Cross *et al.,* 2004).

7.2.1. Biomarkers of exposure to HAs

Dietary studies have shown that HAs are excreted in urine in a dose-dependent manner according to intake in rats (Frandsen *et al*., 2002) and in humans (Stillwell *et al*., 1997). In humans, the major part of the dose is excreted in urine within 24–48 h following a meal; a few percents are excreted as parent compounds, whereas the major parts are metabolites. Thus, urinary levels of parent HAs reflected only recent exposure; however, the pattern of excreted metabolites might indicate the capacity to activate or detoxify HAs (Alexander *et al*., 2002). PhIP is excreted in the urine its major metabolites detected in human urine are *N*2-OH-PhIP-*N*2-glucuronide, PhIP-*N*2-glucuronide, *N*2-OH-PhIP-*N*3-glucuronide, and 4'-PhIP-sulphate with the first two metabolites accounting for most of PhIP excreted (Kulp *et al*., 2000; Malfatti *et al.,* 1999).

In anoher study, eight volunteers were fed 200 g of cooked chicken containing a total of 27 μg PhIP (Kulp *et al*., 2000). Urine samples were collected for 24 h after the meal, in 6 h aliquots; 4-53% of the total ingested PhIP dose was found in the urine. The rate of metabolite excretion varied among the subjects; however, in all of them the majority of metabolites were excreted in the first 12 hours. The variation seen in the total amount, excretion time and metabolite ratio suggested that individual digestion, metabolism and/or other components of the diet may influence the absorption and amounts of metabolic compounds produced from PhIP (Kulp *et al*., 2000). Another study (Strickland *et al.,* 2002) measured the time course of PhIP in untreated and acid- or alkali-hydrolyzed urines from ten healthy non-smoking subjects ingesting identical amounts of charbroiled beef (containing both HAs and PAHs) for five days. The morning after the first day of broiled beef consumption (containing 7.7μg PhIP), urinary concentration of PhIP increased 14 to 38 fold above mean pre-feed concentration; following cessation of broiled meat consumption, urinary PhIP declined to near pre-feed levels within 48–72 h. These results indicated that significant amounts of PhIP were bioavailable from the ingestion of char-broiled beef, and that ratio of conjugated to unconjugated urinary PhIP metabolites may depend on a variety of metabolic, dietary, and hydrolysis factors (Friesen *et al*., 2001; Strickland *et al.,* 2002). In another dietary study (Stillwell *et al*., 1999) 66 subjects ingested a uniform diet of cooked meat containing known amounts of MeIQx, and urine was collected after consumption of the test meal; most of the unconjugated MeIQx was excreted in the urine within 12 hours after consuming the high HAs meals (Stillwell *et al*., 1999).

The variability in the proportion of the dose excreted among the subjects may be reflective of several factors, including inter-individual variation in the enzymatic activity of CYP1A2 and/or conjugation reactions of the N-hydroxylamine metabolite with N-glucuronosyltransferase(s). Linear regression analyses showed that lower total MeIQx (unmetabolized plus the N2-glucuronide and sulfamate metabolites) in urine was associated with higher CYP1A2 activity, whereas total PhIP (unmetabolized plus conjugated) in urine showed no association with CYP1A2 activity. In humans, MeIQx metabolism and disposition were more strongly influenced by CYP1A2 activity than were those of PhIP. Linear regression analysis found no association between NAT2 activity and the levels (unmetabolized plus acid-labile conjugates) of MeIQx or PhIP excreted in the urine (Stillwell *et al*., 1997). In rats, 91% of 5-hydroxy-PhIP was excreted in urine within 24 h and the excretion of 5-hydroxy-PhIP showed a linear dose-response relationship in rats dosed orally with PhIP. 5-OH-PhIP may therefore serve as a biomarker for the formation of the ultimate mutagenic metabolite of PhIP (Frandsen *et al*., 2000; Frandsen *et al*., 2002). At present, the most studied biomarkers of

exposure to HAs are protein adducts in blood as well as HAs in hairs; they reflect long-term exposure (Alexander *et al*., 2002).

7.2.2. Biomarkers of exposure to PAHs

As already mentioned in this chapter, after entering the human body, PAHs undergo a series of biotransformation processes. During Phase I metabolism, they are oxidized by cytochrome P450 enzymes to form highly reactive epoxide intermediates, which are then reduced or hydrolyzed by the epoxide hydroxylase enzyme to hydroxylated metabolites; PAHs can also be directly hydroxylated without formation of the epoxide. In Phase II metabolism, the hydroxy-PAH metabolites are conjugated with glucuronic acid or sulfate to facilitate detoxification and excretion through urine or faeces (Grover, 1986; Kim *et al*., 1998; Pelkonen *et al*., 1982). Several metabolites can be identified from this process, including epoxides, DNA-adducts, dihydrodiols and conjugated monohydroxy-PAHs (OH-PAHs).

Until now, DNA-adducts in blood and OH-PAHs in urine have been used as biomarkers for evaluating PAH exposure. Measurement of PAH metabolites in human urine is the method of choice to determine occupational and/or environmental exposure of an individual to PAHs, in particular, when multiple routes of exposure have to be taken into account (Jacob *et al*., 2002). However, unlike the PAH metabolites excreted in urine which mainly reflect internal exposure, the DNA and protein adducts represent biochemical effects *per se*, which are closer to the cancer risk of the individual than the PAH metabolites excreted in urine (Angerer *et al.,* 1997).

Chapter 2. Colorectal Cancer in Cambodia

1. Introduction

Non-communicable diseases, including cardiovascular diseases, cancers, diabetes and chronic respiratory conditions, represent a major public health threat in Southeast Asia, now responsible for 60% of deaths in the region (Dans *et al*., 2011; Ghaffar *et al*., 2004). Indeed, developing countries in this part of the world are facing large and rapidly growing cancer caseloads for which their health and social service systems must rapidly be prepared. In 2002, 4.2 millions of new cancer cases (39% of new cases worldwide) were diagnosed among 3.2 billions of persons (48% of the world population) living in the fifteen most highly developed countries in South, East, and Southeast Asia: Japan, Taiwan, Singapore, South Korea, Malaysia, Thailand, China, Philippines, Sri Lanka, Vietnam, Indonesia, Mongolia, India, Laos, and Cambodia. China and India, together accounting for 37% of the worldwide population, reported 3 millions of these newly diagnosed cancer cases (McDonald *et al*., 2008).

Cambodia is one of the developing countries in the southwestern part of Indochina, with a land area of 181,035 km^2. In 2008, Cambodian population was around 13.4 millions, with 48.5% males, growing at an estimated rate of 1.54% per annum, and with a life expectancy at birth of 59 and 65 years for men and women respectively (NIS, 2008). In this country, according to the Department of Oncology at the Cambodian-Russian Friendship Hospital, there is no national statistics screening the number of patients with cancer. Yet, evolution of cancer is very worrying in this country, as in recent years, according to the World Health Organization (WHO), the incidence of this disease as well as the number of related deaths have increased. Hence, in Cambodia, chronic diseases accounted for 34% of all deaths in 2002 (55 000), in which cancer killed approximately 6% (3300) (WHO, 2002). By 2030, cancer is expected to be in the top four main causes of death in Southeast Asia as well as in Cambodia (Mathers *et al*., 2005). Lung cancer is the most common or second-most common cancer among males in almost all Asian countries. Stomach cancer is the highest incident rate cancer in both Japan and Korea. Breast cancer among females is the most common or second-most common cancer in all but three of the Asian countries (China, Mongolia, and Thailand). Stomach cancer is the highest incident cancer among females in China and Korea; uterine cervix cancer is the highest in Cambodia, India, Laos, Thailand, and Vietnam (McDonald *et al*., 2008).

Colorectal cancer (CRC) is the most common malignancy of the gastrointestinal (GI) tract. It is the third most common cancer in men and the second most common cancer in women worldwide (Ferlay *et al*., 2010). CRC is generally thought of as a disease of older persons, with more than 90% of patients being diagnosed after the age of 55 years (Atkin *et al*., 2010). It is well known, however, that CRC also affects a young population under 40 years of age (Meyer *et al*., 2010; O'Connell *et al*., 2003; O'Connell *et al*., 2004). Many Asian countries, including China, Japan, South Korea, and Singapore, have experienced a two to four times increased incidence of CRC during the past few decades (Ferlay *et al*., 2007; Wong *et al.,* 2007). The rising trend in incidence and mortality from CRC is more striking in affluent than in poorer societies, and differs substantially among ethnic groups (Sung *et al*., 2005). Although changes in dietary habits and lifestyle are believed to be the reasons underlying this increase, the interaction between these factors and genetic characteristics of the Asian populations might also have a pivotal role (Huxley *et al*., 2009; Sung *et al*., 2005; Yee *et al*., 2009).

Epidemiologic features of CRC in Cambodia have not been studied adequately yet. The aim of this study is thus to present the first comprehensive report on the incidence of this disease in that country, giving epidemiological, clinical and pathological characteristics of CRC in Cambodia. For that purpose, we used the clinical and pathological data on all primary CRC cases diagnosed between 2005 and 2010 that were obtained from the Reference Center for Gastro-intestinal Tumours Surgery and Marie Curie Cancer Center (Cambodian-Russian Friendship Hospital) in Cambodia. The incidence of CRC and the age specific incidence rate have been calculated using the collected data from this centre.

2. Material and Methods

2.1. Cancer Registry and CRC Data Collection

Cambodia is one of the developing countries in the southwestern part of Indochina. In 2008, the Cambodian population was around 13.4 millions of persons (48.5% males), with an estimated growing rate of 1.54% per annum, and a life expectancy at birth of 59 and 65 years for men and women respectively (NIS, 2008). Cancer registries play an indispensable role in population surveillance, risk analysis, policy planning and finally, completion. The Cambodia registry of cancer was created in 2003 in the Reference Center for Gastro-intestinal Tumours Surgery and the Marie Curie Cancer Center, both of them located in Khmer-Soviet Friendship Hospital in Phnom Penh, the capital city of Cambodia. For diagnosis and mainly for treatment, patients were referred from all provinces in Cambodia and from both private and public hospitals. There is no other center

offering standard cancers treatment in this country. Clinical and pathological data on all primary CRC cases diagnosed between 2005 and 2010 were obtained from this database of the Reference Center for Gastro-intestinal Tumours Surgery and the Marie Curie Cancer Center. The data collection in 2003 and 2004 did not enter for CRC incidence data analysis because they were not completed selection criteria. All the information available in the center including clinical, pathological, therapeutic and evaluative data were collected and analyzed with the aim to identify prognostic factors.

2.2. Data treatment

For CRC and other cancers, age-specific incidence rates per 100 000 were computed based on cancer data mentioned previously and Cambodian population census 2008 data from the National Institute of Statistics (**Annexe II**) (NIS, 2008).

We compared the age-specific of CRC with the other cancers, and the differences in distribution of colorectal tumour location and histology. The differentiation of tumour location in two age groups, namely < 40 and ≥ 40 years old was compared using SPSS 19.0 statistical software system (SPSS Inc., Chicago, IL, USA) for all analyses. A study of patients' survival was not possible due to very limited follow-up data.

3. Results

3.1. CRC incidence in Cambodia

According to the data collected from the Department of Oncology at the Cambodian-Russian Friendship Hospital (Gastro-intestinal Tumours Surgery and the Marie Curie Cancer Center), among cancer patients who came for diagnosis and treatment in this hospital since 2003 (starting year of cancer registration in Cambodia), almost a quarter had cervical cancer (25%), followed by breast cancer, and nose, ear or throat cancer (around 15%) mainly in men who smoke and drink. **Table 2.1** presents the number of new cases and age-standardized of the top five cancers in Cambodia. Before 2009, CRC has been classified as 5th range of the top five common cancers in Cambodia. In 2009, CRC has been classified in 4th range as shown in Table 1. The details related to CRC are indicated in **Table 2.2**.

Table 2.1. The most common cancers reported in Cambodia.

New cases	2005	2006	2007	2008	2009	2010
Cervical cancer	146	198	191	232	212	NA
Nose, ear or throat	119	118	113	108	165	NA
Breast	66	115	133	130	148	NA
CRC	31	37	45	62	95	76
LMNH/LH	23	55	56	67	57	NA
Incidence rate per 100 000*	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**
Cervical cancer	1.07	1.45	1.40	1.70	1.55	NA
Nose, ear or throat	0.87	0.86	0.83	0.79	1.21	NA
Breast	0.48	0.84	0.97	0.95	1.08	NA
CRC	0.23	0.27	0.33	0.45	0.69	0.56
LMNH/LH	0.17	0.40	0.41	0.49	0.42	NA

LMNH/LH: Non-and Hodgkin lymphoma/Hodgkin lymphoma cancer
NA: not available
**Incidence rate per 100 000 was calculated based on the Cambodian population census 2008 data from the National Institute of Statistics (NIS, 2008).*

Table 2.2. Evolution of CRC in Cambodia for the period 2005-2010.

CRC/Sex		2005	2006	2007	2008	2009	2010	Total
Male	New cases	15	13	13	33	42	38	154
	Incidence rate per 100 000	0.23	0.2	0.2	0.51	0.64	0.58	2.36
Female	New cases	16	24	32	29	53	48	202
	Incidence rate per 100 000	0.22	0.34	0.45	0.45	0.74	0.67	2.82

From 2005 till 2010, the total number of new cases of CRC was 356. Incidence rates per 100 000 were 2.36 and 2.82 in men and women, respectively. Overall, the CRC incidence seems to increase from the year when diagnosis started, with a tendency of a higher increase in women than in men; the highest incidence was observed in 2009 in both sexes with incidence rates of 0.64 and 0.74 per 100 000 for men and women, respectively.

3.2. CRC characteristics in Cambodia

In Cambodia, the mean age at diagnosis was 49 ± 16 years considering the 2005-2010 periods. Female patients constituted near 57% (n=202) of the total CRC cases, with a female-to-male ratio of 1.31. Overall, the incidence rates increased with age. The values for males were comparable to that of females until the age of 55 years; women had a peak of CRC incidence in the 6th decade (55-64 years old) followed by a second peak in the 7th decade (65-74 years old), whereas men had the highest risk of developing CRC in their 7th decade of life (**Figure 2.1**).

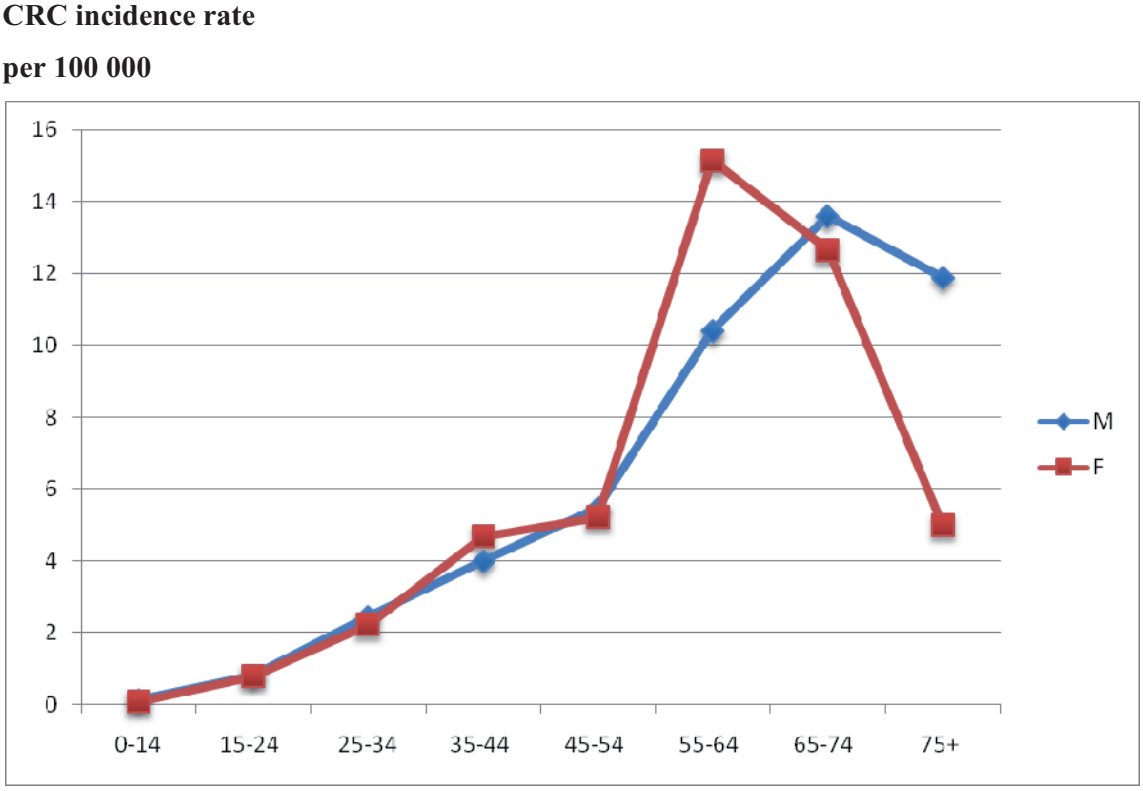


Figure 2.1. Age-specific CRC incidence rates per 100 000 in Cambodia, for both sexes (M: male, F: female), over the 2005-2010 period.

Figure 2.2 shows the age distribution of Cambodian population based on the census data 2008 from the institute of statistic of Cambodia (NIS, 2008). The population of younger age (0-40 years old) represents about 76.7%, with children less than 14 years old (0-14 years old) representing about 33.7% of the total population. The Cambodian population has an estimated life expectancy at birth of 59 and 65 years for men and women, respectively. Consequently, the older persons (over 65

years old) represent only 4% of the total population, being 3.5 and 5% for men and women respectively.

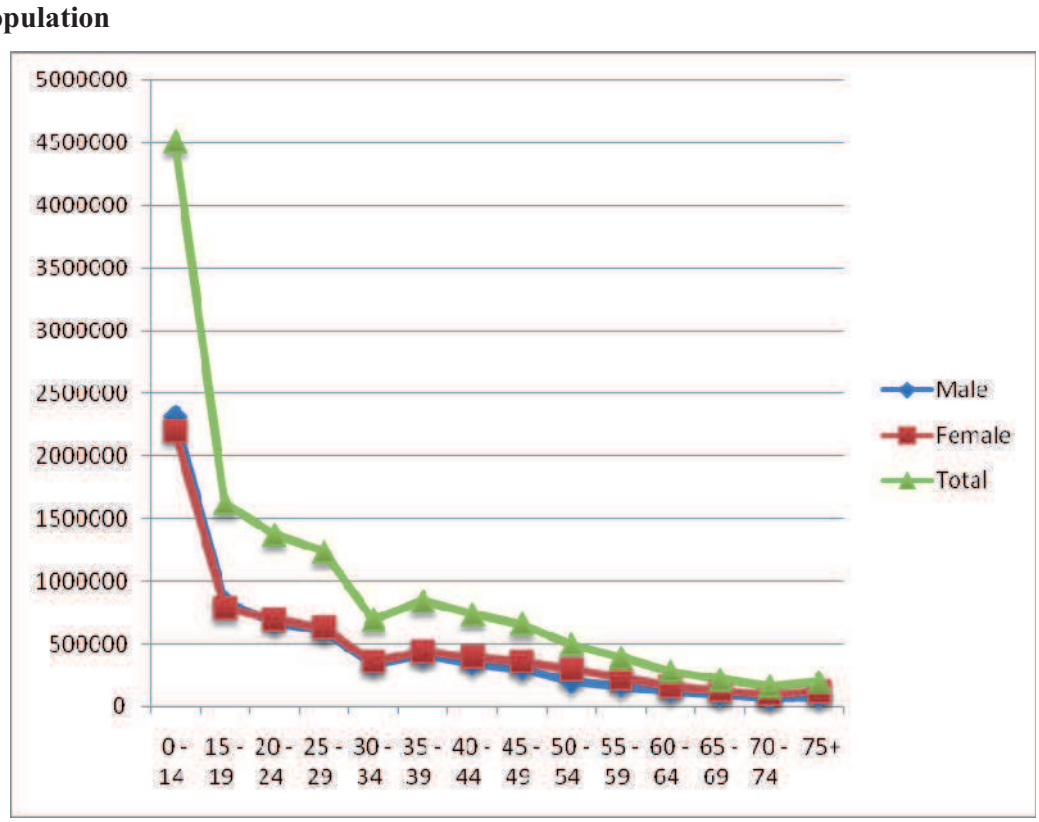


Figure 2.2. Age distribution of Cambodian population, census 2008 data from the National Institute of Statistics (NIS, 2008).

Between 2005 and 2010, among the 356 newly diagnosed CRC cases, 106 (29.8%) affected patients younger than 40 years. This proportion of young age people affected by CRC in Cambodia is the second highest among the rates reported for CRC in Asia, Europe, USA, the Middle East and New Zealand for the same age group (**Fig. 2.3**). However, as previously shown, the Cambodian population is characterized by a high proportion of young population, which could partly explain such an observation.

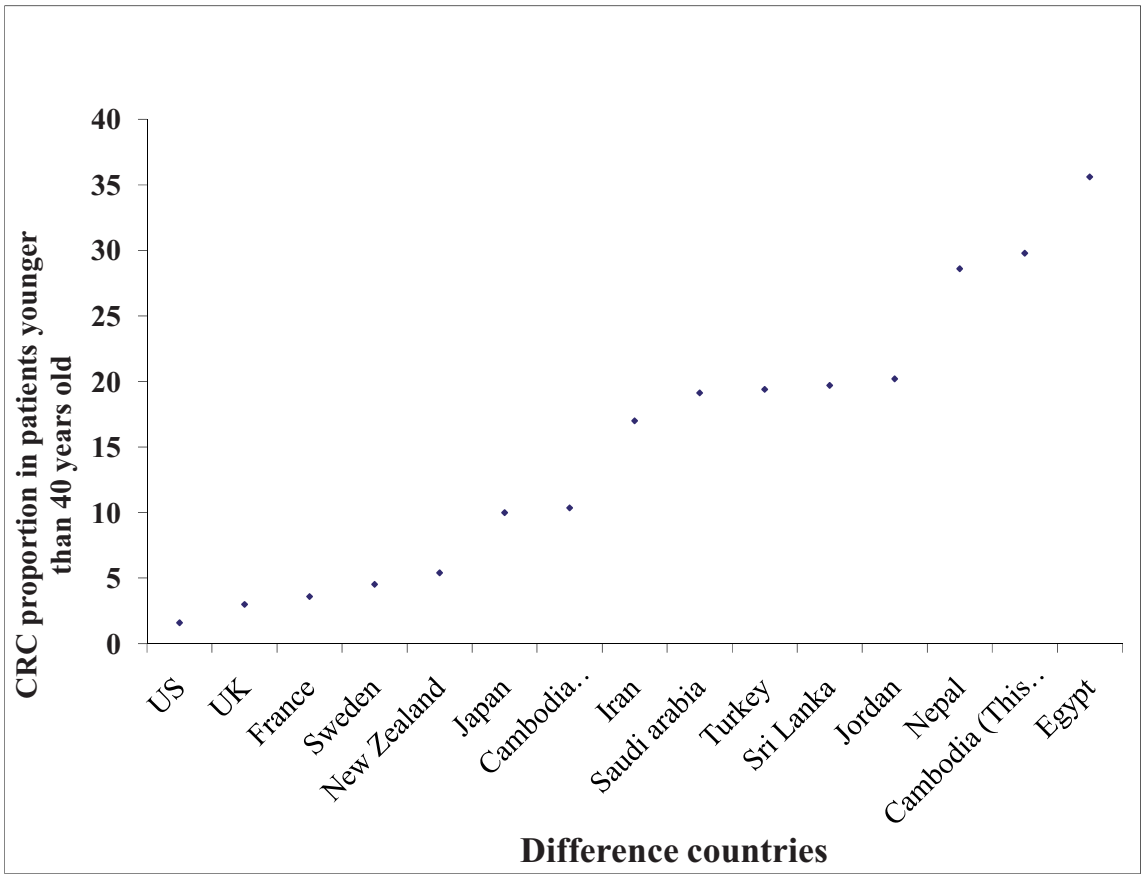


Figure 2.3. Comparison of CRC proportion (%) in patients younger than 40 years in USA, New Zealand and different countries in Asia, Europe and the Middle East.
[Griffin *et al*., 1991 (USA). Adloff *et al*., 1986 (UK). Domergue, 1988 (France). Ohman, 1982 (Sweden). Ibister, 1990 (New Zealand). Okuno, 1987 (Japan). Ferlay *et al.,* 2010 (Cambodia, Globocam); Ansari et al.,, 2006 (Iran); Isbister, 1992 (Saudi Arabia); Yilmazlar *et al*.,1995 (Turkey); Al-Jaberi *et al*., 2003 (Jordan); Hav *et al*., (Cambodia, this study); Soliman *et al*., 1997 (Egypt)].

Table 2.3 presents the pathologic characteristics and colorectal tumour subtype of the 356 CRC cases reported in this study. For all ages, adenocarcinoma was the most common histological type, ranging from 91.7 to 96.4% of CRC cases for age groups younger and older than 40 years respectively. More than one half of all tumours occurred in the colon, with the same distribution in men (63%) as in women (62.4%); no appreciable variation of tumor location between the two age groups was observed, as the incidence rates of right colon (ascending) and left colon (descending) cancer for both genders was found to be similar. The proportion of cancer ascending colon, transverse colon and descending colon was 33.8%, 7.4% and 25 % for patients younger than 40 years old, and 32.6%, 1.8% and 41.8% for the patients older than 40 years old, respectively. In the total of 133 new reported cases of rectum cancer, 42.9% were found in men and 57.1% in women.

As for the colon cancer, the distribution of rectum cancer between both groups (younger and older age than 40 years) had nearly the same percentage.

Table 2.3. Gender, type and location distribution of CRC reported cases in Cambodia over the period 2005-2010.

Gender / Type / Location	**Total**	**Age groups**	
		< 40 years, n (%)	**≥ 40 years**, n (%)
Gender	356	106	250
Female		56 (52.8)	146 (58.4)
Male		50 (47.2)	104 (41.6)
Tumour histologic type	356	106	250
Adenocarcinoma		97 (91.5)	241 (96.4)
Non-adenocarcinoma		9 (8.5)	9 (3.6)
Location	356	106	250
Colon		68 (64.2)	155 (62.0)
Rectum		38 (35.9)	95 (38.0)
Colon	223	68	155
Descending colon		17 (25.0)	65 (41.8)
Ascending colon		23 (33.8)	50 (32.6)
Transverse colon		5 (7.4)	3 (1.8)
Colon, unspecified		23 (33.8)	37 (23.8)

4. Discussion

4.1. Low CRC incidence in Cambodia

CRC is the fourth most common cancer in the world and the second most common cancer causing death in the developed countries with a western culture such as United States, New Zealand, Australia, Canada, European countries and in several Asian developed countries such as Japan, South Korea, or Singapore (Ferlay *et al*., 2010; Ferlay *et al*., 2007; Sung *et al*., 2005). Although it is generally believed that CRC is more prevalent in Western countries, there has been a rapid rise over the past few decades in CRC incidences in more developed countries in Asia and Asia-pacific region (Sung *et al*., 2005; Sung *et al*., 2008; Chen *et al*., 2002).

CRC in Cambodia is the fourth cancer in frequency after the cervical cancer, nose / ear or throat cancers, breast cancer, and LMNH/LH cancer. Over the period 2005-2010, the estimated incidence rate of CRC in Cambodia is 2.6 for both sexes, 2.82 for men and 2.36 for women (see **Table 2.2**). This incidence rate is lower when compared with other next countries like Thailand (8.8 for males and 7.6 for females), Japan (41.7 for men and 22.8 for women), Singapore and South-Korean (46.9 for men and 25.6 for women) (Ferlay *et al*., 2010; Khuhaprema *et al*., 2008; Leung *et al*., 2006; Wong *et al*., 2007). Most cases of CRC were diagnosed at an advanced stage, with adenocarcinoma being the most common histological type.

Temporal trends in incidence are related to three main factors: age, period of diagnosis and birth cohort. Study of CRC using age-period cohort models is commonly used in order to better understand the observed trends and aetiological factors connected with them (Clayton *et al*., 1987a,b). The number of reported cases of CRC in both sexes remains similar in Cambodia for the last two years. Over a 6-year period (2005-2010), we have observed that the overall incidence of CRC in Cambodia has slightly increased in both males and females.

CRC was long considered to be a homogeneous entity, but several studies suggest that its characteristics differ by anatomical subsites (Faivre *et al*., 1989; Johansen *et al.,* 1993). However, availability of incidence rates by subsites is rather limited. In the USA, stable incidence rates for right colon cancer, and declining rates for left colon (descending and sigmoid) and rectal cancer were reported over the 1992-2001 period (Cress *et al*., 2006). Evidence exists for different time trends. Among available data in Europe, an increase in all colon subsites was reported in Denmark (Clayton *et al*., 1987a; Thygessen *et al.,* 2004), Norway (Svensson *et al*., 2002) and Italy (Capocaccia *et al*., 1997; Ponz de Leon *et al*., 2007). A recent study from Norway reveals deceleration in the rate of increase of colon cancer subsites (Larsen *et al*., 2010). In most high-risk areas of CRC (western countries and/or developed countries), the incidence of rectal cancer decreased with time (USA, Denmark, France) or was stable (Italy, Australia), whereas it increased in Norway (Larsen *et al*., 2010; Thorn *et al*., 1998). In low-risk areas of Asia, Central and South America, there was a striking increase in incidence of all colon subsites, while it was moderate for rectal cancers (Curado *et al*., 2007; Parkin *et al*., 2003). We may assume that this marked increase in such developing countries is partly due to the fact that new cases of CRC are now better detected and registered as it was years ago. Among other factors that may explain such an observation, the changes in life-style, especially in food diet, is also to be considered.

4.2. CRC younger age in Cambodia

The likelihood of colorectal cancer diagnosis increases after the age of 40, increases progressively from age 40, rising sharply after age 50 (WCRF, 2007; Haggar *et al*., 2009). More than 90% of colorectal cancer cases occur in people aged 50 or older (Haggar *et al*., 2009; NIH, 2006). The incidence rate is more than 50 times higher in persons aged 60 to 79 years than in those younger than 40 years (Haggar *et al*., 2009). Although CRC is a common disease in the aging population, it has been evident since the largest systemic review that the disease is not infrequent in the young or rare before the age of 40 years (O'Connell *et al*., 2004; WCRF, 2007). In this study, CRC proportion in patients aged less than 40 years (29.8%) is approximately four times higher than described in developed countries, and is the second highest rates reported in Asia and the Middle East (see **Fig 2.3**) (Al-Jaberi *et al*., 2003; Ansari *et al*., 2006; Isbister *et al*., 1992; Soliman *et al*., 1997; Yilmazlar *et al*., 1995).

Most of the studies on CRC observed in young people have looked at clinical pathological features of tumours as well as patients' prognosis and survival. However, very few studies have described the risk factors for development of CRC at such young age. Among the three well documented categories of predisposing conditions, namely dietary (especially western style diet), environmental and genetic, the last one has been most frequently found in the young population (Corrêa Lima *et al*., 2005; Haggar *et al*., 2009).

Two principal forms of hereditary CRC are familial adenomatous polyposis (FAP) and Lynch syndrome. The latter, also called hereditary non-polyposis colorectal cancer (HNPCC), is a common autosomal dominant syndrome characterized by microsatellite instability and early age at onset of neoplastic lesions in a variety of tissues (e.g., endometrial, gastric, renal, ovarian, and skin). FAP, accounting for < 1% of CRC, is also an autosomal dominant disease classically characterized by the development of hundreds to thousands of adenomas throughout the rectum and colon during the second decade of life. Classic FAP results from a germline adenomatous polyposis coli (APC) mutation (Half *et al*., 2009; McCart *et al*., 2008). A subset of individuals with clinical features of FAP will instead carry a mutation in the MUTYH gene (Sampson *et al.,* 2005). Individuals with HNPCC and FAP carry about 80 and 100% risk of developing CRC, respectively (Hampel *et al*., 2000). MacGillivary *et al.* (1991) observed HNPCC in young adults in 2% of their CRC cases, while FAP was reported in 8.1% (Martin *et al*., 1981) and 10% (MacGillivary *et al*., 1991) of young patients. Although these genetic changes have not been tested in Cambodian CRC, we speculate that genetic alterations leading to HNPCC might be more common in Cambodian population.

Family history and parental consanguinity have been demonstrated to be strongly associated with CRC development in Arab population (Bener *et al*., 2010). Even though family history of our CRC patients has not been studied, consanguinity, which is also prevalent in Cambodian residents, can be a contributing factor in increasing the risk of early CRC onset in this population.

CRC incidence rates are increasing in countries that are undergoing rapid industrialization because of the lifestyle risk factors including diet, physical activity, and obesity, which play an essential role in the etiology of the disease. Peters *et al*. (1989), investigating the diet of individuals under 45 years of age in California, found that deep fried foods as well as barbecued or smoked meats increased the risk of cancers of the caecum and ascending colon in this subset of population (Peter *et al*., 1989). Hence, investigating the possible effect of diet on CRC incidence rate in Cambodia will be one of the objective of the study conducted in this work (see **Chapter 3**).

Environmental factors such as exposure to pesticides and other chemical substances have been also associated with CRC in young adults. Pratt *et al*. investigated a cluster of 13 adolescents with a history of exposure to agricultural pesticides and other chemicals used in growing cotton, rice and soybeans on southern US farms. Nine of 13 patients had exposure to farm or agricultural chemicals (Pratt *et al*., 1977). In Cambodia, according to the population census 2008, about 75% of Cambodians are engaged in agriculture. Highly toxic pesticides belonging to WHO class I + II and banned or restricted by the Cambodian law are still widely used in this country (Jensen *et al*., 2011). This could be one of the factors contributing to the early occurrence of CRC in Cambodian population.

Regarding tumour location and histology, our findings that more than one half of the CRC cases were diagnosed in the colon and that adenocarcinoma was the most common histologic type are in agreement with most studies (O'Connell *et al*., 2004). Moreover, we found that mucinous and advanced-grade tumours were twice more frequent in the younger age group than in the older. More than one half of the CRC patients studied here presented an advanced disease. This could be explained by the lack of CRC screening in Cambodia. There was no significant difference between the numbers of young and old patients presenting an advanced CRC, but information on this parameter were available for only 41% of cases. Most of the series regarding young colorectal patients agreed on the fact that the disease is presented at an advance stage (Kam *et al*., 2004; Liang *et al*., 2003; O'Connell *et al*., 2003; Yiu *et al*., 2004). In comparison, more patients having the early stages of the disease belong to the older age group (El-Hennawy *et al*., 2003; O'Connell *et al*., 2003; Rasul *et al*., 2001). Another significant difference documented between the younger and

older age groups is the occurrence of the mucinous subtype of adenocarcinoma, which is more prevalent in the patients below 40 years of age (Al-Jaberi *et al*., 2003; Kam *et al*., 2004; Liang *et al*., 2003; Singh *et al*., 2002).

The elevated proportion of young age people affected by CRC in Cambodia may be also partly explained by the high proportion of this young population (76.7% of the total population was younger than 40 years in 2008). The same finding has been highlight by Ansari *et al*. (2006). We found that younger people present later stage disease and poorer tumor grades at diagnosis (Palmer *et al*., 1991). This could be explained by the fact that young patients are not screened (O'Connell *et al*., 2003) or are at increased risk because of a higher prevalence of conditions predisposing them to CRC. Differences in stage and grade of disease among younger cases may also be attributable to delays in diagnosis caused in part by delays in patient presentation or lack of access to medical care. Hence, studies have reported delays in presentation as long as nine years, mostly due to patient factors such as lack of knowledge about CRC symptoms, particularly among persons younger than 50 years (Lee *et al*., 1994; O'Connell *et al*., 2004; Parramore *et al*., 1998), emphasizing the need for increased awareness about CRC incidence in persons younger than 50 years. Providing education on the CRC symptoms and signs to patients before the recommended screening age of 50 years may decrease the delays in diagnosis and subsequently positively affect the stage of disease at diagnosis.

5. Conclusion

In summary, we found that approximately 30% of Cambodian CRC patients were younger than 40 years. For the same age group, this proportion is remarkably higher than that estimated by GLOBOCAN 2008, in which CRC rates in Cambodia were computed based on observed cancer rates in Thailand (Ferlay *et al*., 2010). It can be speculated that the unusually high CRC proportion in the young observed in our study might result from referral bias. Hence, although the two referral centers received CRC patients from all provinces, the unfavorable economic status and attitude of most Cambodians allow us to assume that many old people diagnosed with CRC elsewhere might have refused treatment and that some other patients, particularly those living along the borders with Thailand and Vietnam, might have gone for treatment in these two countries. Nevertheless, this peculiarly high proportion of early-onset CRC, the continuous exposure to hazardous environmental agents, and the prevalent consanguinity in Cambodia justify further research, which will advance our understanding of the risk factors for the disease in young adults. These studies should investigate environmental exposures, family history, and consanguinity as well as explore gene–environment interactions in CRC carcinogenesis in this high-risk population.

Chapter 3. Dietary patterns, energy and nutrients intake in Cambodia

In nutritional epidemiology, researchers often investigate food(s), or nutrient(s) in the association between exposure and disease and diet has been shown to be associated with many chronic diseases, such as coronary heart disease, obesity, diabetes, and cancer (FAO/WHO, 2003). Several studies have indicated a role of diet in the etiology of colorectal cancer (Giovannucci *et al*., 2006; Newby *et al*., 2004). Most studies have investigated the effect of specific foods, food groups, or nutrients but the analysis of the whole dietary patterns has thus been proposed to understand the association between diet and colorectal cancer (Hu, 2002).

Accordingly to these authors we conducted a study on diet, energy and nutrients intake for the Cambodian population including the well known and usual suspects in the etiology of CRC in order to possibly understand the protective effects of the current Cambodian diet as well as to establish a point of departure for future monitoring. We looked for risks and benefits of dietary pattern (i.e., food groups, food items, macronutrients, micronutrients, meat doneness level, quantity, frequency and cooking methods) as well as obesity in 941 Cambodian people, aged from 25 to 65 years, in four different regions of the country including the Capital city.

1. Introduction

The ongoing nutritional transition in Asia is marked by a shift away from relatively monotonous diets of varying nutritional quality towards an industrialized diet, usually more varied, which includes more pre-processed food, more food of animal origin, more added sugar and fat, and often more alcohol (Amuna *et al*., 2008; Popkin *et al*., 2001). South Asia malnutrition (poor nutrition) and under nutrition (inadequate nutrition) are closely associated with poverty, with 21% of people suffering from severe energy deficiency (Smith *et al*., 2007; Tuyen, 2009). Diet-related chronic diseases are projected to increase, and dietary factors will account for an increased share of chronic diseases (principally obesity). The experience from Thailand, with the rapid increase in fat consumption and changing lifestyle patterns, clearly demonstrated a significant impact on the shifting pattern of disease burden of the population (Kosulwat, 2002; Kwanbunjan *et al*., 2005).

Dietary intakes, dietary habits and cooking practices are very specific from country to country and region to region, being particular to each civilization. The dietary habits of a population are influenced by many factors, including the availability of food, level of income, health, food beliefs,

dietary laws, as well as religion and cultural patterns, customs; additional factors include age (in particular, generation), region of origin, and occupation (Carlson *et al*., 1984; Devine *et al*., 1999).

In Cambodia around 80.5% of the total population lives in rural areas. The capital city, Phnom Penh, has a population of 1.3 millions. The country shares a border with Thailand to its west and northwest, with Laos to its northeast, and with Vietnam to its east and southeast. The geography of Cambodia is dominated by the Mekong River and the Tonlé Sap (Great Lake) as an important source of fish (NIS, 2008). There is no statistical data on nutrition, nor food consumption and behavior of the general population in Cambodia. The most recent information concerning the diet of the Cambodian population has come from studies of the Institute of Statistic that focused on women and children, including the lactating and pregnant women and malnutrition in children less than 5-year old (NIS, 2006). The assessment of food consumption in such studies was based on the calculation of expenditure and income of non-food items. Currently, there are several international organizations like WHO, Food Agricultural Organization (FAO) and UNICEF which are interested in studies of micronutrient deficiency and poor nutrition related to children under five years (NIS, 2006). The amount of food consumed was not measured by the surveys and most consumption surveys were rather qualitative. The Cambodia socio-economic survey as well as the food insecurity assessment of the magnitude of hunger (as measured by the prevalence of food limitation) in 2003/2004 approximated that one person out of five suffers from malnutrition in Cambodia. Higher levels of nutrient deficiency were observed in people with low incomes and in rural areas (NIS, 2007). Women also seemed to be affected by malnutrition. According to the UNICEF survey, the prevalence of 15 to 49 years old women with a body mass index lower than 18.5 kg/m^2 was 28.5% (FAO, 1999). However, the proportion of the population undernourished in Cambodia declined from 43 to 26% between 1990 and 2005 respectively (Tuyen, 2009).

There is a lack of data on the dietary intake, dietary behavior and nutritional status in the Cambodian population. Our study is the first one to assess the dietary patterns of the Cambodian people in both rural and urban areas, and to describe and analyze the food groups contributing to overall consumption. We also tried to assess possible differences in dietary intake between the wet and dry seasons in four different regions of the country. Results of this work will provide the first database of food consumption in the Cambodian population, which is crucial for scientists working in the fields of nutrition, food security and food safety.

2. Material and Methods

Adapted to the situation in Cambodia and to the objectives of our research, 24-hour recall and food frequency questionnaires were suitable methods to use in this survey (Biro *et al*., 2002; Feunekes *et al*., 1995).

2.1. Establishment of relevant questionnaires

2.1.1. 24-hour recall questionnaire

Food consumption varies considerably from country to country and/or region to region due to variations in ethnicity, geographical areas, income, age and sex. Therefore, the development of a standard questionnaire must adapt to the circumstances of each research. For our study, food consumption data were obtained using the 24-hour recall, which included, in addition to personal data, indicators of social economic level (education, occupation, family, household information and physical activity). The 24-hour recall repeated twice is one of the most cost-effective methods for assessment of dietary intake (Biro *et al*., 2002; Hoffmann *et al*., 2002). The 24-hour recall questionnaire used consisted of 250 food items with 14 food groups: cereals and products; starchy roots and tubers; legumes, nuts and seeds; vegetables and products;, fruits and products; meat and products; fish, shellfish and products; eggs and products; milk and milk products; fats, oils and products; sugar and confectionary; condiments and spices; alcoholic beverages; and finally non-alcoholic beverages, based on the ASEAN Food Composition Table (Puwastien *et al.,* 2000). Composite dishes were analyzed as normally consumed, without extracting added oil, fats, salt, sugar or other ingredients from recipes. The ingredients intake collected parallel with 24-hour recall were added into the database (see Annexe III).

2.1.2. Food frequency questionnaire

Dietary habits and cooking practices of several specific foods were monitored with a specifically designed Food Frequency Questionnaire (FFQ) (**see Annexe III**). FFQ was developed and pre-tested in a feasibility study to comply with the Cambodian situation; it has been improved for its final use in this survey. The questionnaire contained a list of 100 different items of red meat, poultry, fish, sausage, and offal products. Four different cooking methods (grilled, pan-fried, deep-fried and baked) were included, along with four different degrees of doneness (rare - R, medium - M, well done - WD, and very well done - VWD). In this questionnaire, the five categories of intake

frequencies ranged from one time per day (1T/D), one time per week (1T/W), two times per week (2T/W), three times per week (3T/W) and one time per month (1T/M). The FFQ was administered by face to face interviews where subjects were asked about their usual consumption, frequency of various types of meat, poultry, sausage, offal products and fish, including portion size, method of cooking and degree of doneness for each of the food items listed.

Subjects were also requested to declare the number of servings consumed each time they ate the food. Several food pictures with three different portion sizes and level of doneness were used during the survey as detailed below. The participants were also asked to describe the method for food preparation (marinated and/or dry) before cooking; each subject was required to choose only one option. Participants were also asked for their weight and height, this information being used to calculate their body mass index (BMI).

2.2. Development of food portions and pictures

To be able to quantify food consumption, as well as intakes of nutrients or other food constituents, a measure or estimate of the portion size of each food item consumed is required. Several methods of measuring dietary intake have been used. The estimation of portion sizes from food photographs has been used widely in many types of studies, and it was useful for measuring dietary intake for population-based studies (Foster *et al*., 2005; Godwin *et al*., 2004). So, we decided to also use this method in this survey.

For that purpose, main foods known to be eaten in Cambodia (including traditional foods and dishes) were cooked. Then they were presented into three difference portion sizes (small, medium and big) according to command portion size consumption. The weight of each portion size has been recorded and photographs of these portions were then taken as illustrated in **Figure 3.1**. These pictures were further recorded in a book, which were used by each interviewer. The measurement of average weight of fruits and vegetables was also carried out and edible part of them was recorded. Finally the weights and photos of small, medium and large portions were obtained for all foods (**Annexe III**).

A B C

Figure 3.1: Examples of pictures obtained for traditional Cambodian food, and used in the questionnaire to help quantifying the food intake: (A) small size portion, (B) medium size portion, (C) big size portion.

2.3. Selection of subjects

The survey was based on a random sampling of subjects. To incorporate the effects of seasonality, the studies of food consumption were made during two seasons. The study population consisted of a total of 941 adult participants with age ranging from 25 to 65 years old. The participants consisted of 603 females and 338 males. For the dry season, the survey took place in March 2010 and involved 500 individuals; for the wet season, it took place in September 2010 with 441 participants. Participants were randomly selected from the four main regions of the country (**Fig 3.2**), namely the capital Phnom Penh (R1), the region around Mekong River and/or near the border of Vietnam (R2),

the geographical region around Tonlé Sab lake and near the border of Thailand (R3), and the region near the sea (R4). The survey area in each region was done by random selection. The study covered at least one urban and several rural areas in each region, in order to have an adequate sample of subjects. The participants were identified by age, sex and areas (rural / urban). They were interviewed two times with an interval of three days between interviews. To avoid the effects of clusters, only one member per considered household was interviewed. A total of 1120 subjects were involved for the first questionnaire; among them 941 (84%) completed the second questionnaire three days after the first one.

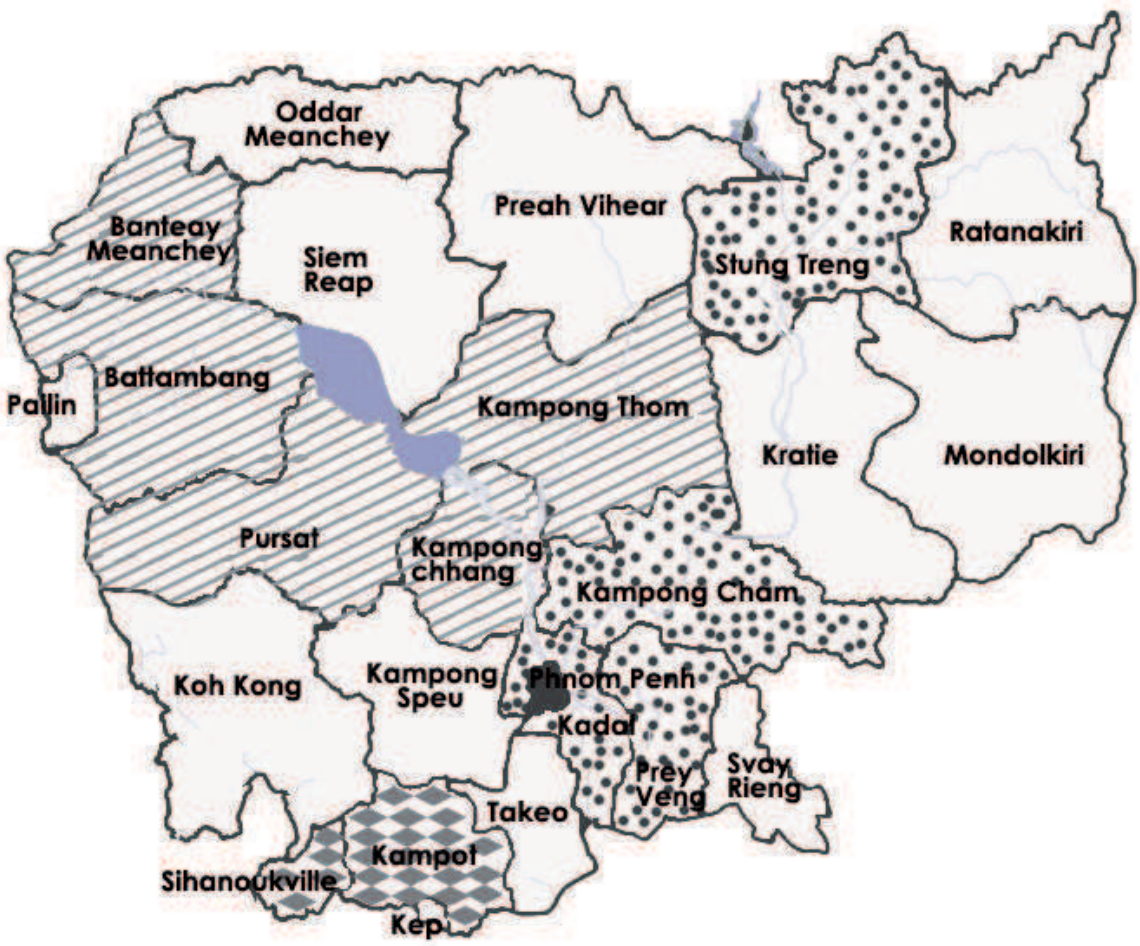


Figure 3.2: Map of Cambodia featuring the four regions of surveys.

R1 ■ R2 ▩ R3 ▨ R4 ▩

2.4. Study design

The participants were invited to reply face to face to socio-economic and demographic information including physical activity, 24-hour recall questionnaire, and FFQ. During the interview, the dietary intakes at home and outside (work place, restaurant, etc.) for the previous day were reviewed, the subject using his memory to provide the answer. Food consumption was recorded from the time the subject woke up until the time he went to bed. It included the three main meals: breakfast, lunch and dinner, as well as the three inter-meal periods (between breakfast and lunch, between lunch and dinner, and after dinner). Detailed description of all food and beverages (including local and brand names), and their method of preparation (dry, marinated) and cooking (fried, stewed, boiled) were recorded. For composite dishes, the amount of each ingredient used in the recipe and the amount consumed by the subject were also recorded. Colored photographs of three different portion sizes of foods were provided to help estimate the quantity of food consumed (see **Fig 3.1**). The whole questionnaire, including the socio-demographic information, 24-hour recall and FFQ required approximately 40 to 60 minutes to be completed.

Interviewers were students of the Institute of Technology of Cambodia (ITC) in Food Chemistry Department. They were previously trained to understand the purpose of the survey and to use the questionnaire adequately; they also pilot-tested it before data collection began. A total of 16 interviewers were divided into four groups, each group being in charge of one region. Interviews were conducted during one week. Each interviewer needed to select and work on about 10 subjects per day. The first round of the survey was completed during the first three days; the second round of the survey started on the fourth day, after the first round was finished. The interviewers went back to meet the same participants they had met at the first, second and third days and did the second interview.

2.5. Data preparation

As already indicated, complete information on diet and socio-economic characteristics was obtained from 941 adults (84% response rate).

2.5.1. 24-hour recall data preparation

As mentioned previously, food questionnaires used in the survey were 24-hour recall questionnaires and FFQ repeated twice. All were repeated at each of the two seasons. After data collection, the

answers were reviewed, corrected and harmonized for accuracy. Body mass index was calculated by dividing weight by squared height (kg/m²). The 24-hour recall data were entered into the Excel database, including detailed percentages of food components in each meal based on food photographs, food ingredients recorded during the survey, and the Khmer food recipe reference (Long *et al*., 2010). The amounts consumed for each food were converted into grams.

Energy and nutrient intakes (including carbohydrates, proteins, total fat, cholesterol, total vitamin A, thiamin, riboflavin, vitamin B6, vitamin B12, vitamin C, vitamin E-tocopherol equivalents, calcium, and iron) were measured using the 24-hour recall method, and calculated by using the ASEAN Food Composition Tables (Puwastien *et al.,* 2000) as well as the French Food Composition Table (Ciqual, AFSSA: http://www.afssa.fr/TableCIQUAL/). The information was then entered into the Excel databases. The macro and micronutrient intakes reported in the present work are based exclusively on the contribution of foods and fluids consumed, and do not include contribution from vitamin and mineral supplements. Mean intake of nutrients was compared with the regional Recommended Dietary Allowances (RDAs) or Dietary Reference Intakes (DRIs).

2.5.2. Food Frequency Questionnaire data preparation

Data obtained from the FFQ were used to determine the consumption frequency of meat and fish products, portion sizes, cooking techniques and doneness levels. To do so, we first evaluated the consumption of meat, poultry, fish, sausage and offal products using frequency and portion size. Then the consumption of these products (expressed in g/person/day) was computed by summing across items that contributed to each food group, and characterized by mean value and standard deviation.

The conversion of food frequency to the amount of food intake was carried out using the following formula (Norimah *et al*., 2008).

:*Amount of food (g/day) = frequency of intake (conversion factor) x total serving weight*

Total serving weight = total number of servings x weight of food in one serving

The conversion factor used to estimate food intake was based on frequency of intake as indicated in **Table 3.1**

Table 3.1. Conversion factors used in this study based on frequency of intake.

Frequency of intake		Conversion factor
Per day	1	1
	2	2
	3	3
Per week	1	0.14 (1/7)
	2	0.29 (2/7)
	3	0.43 (3/7)
Per month	1	0.03 (1/30)
	2	0.07 (2/30)
	3	0.1 (3/30)

Example of calculation to estimate the intake per day based on the frequency of intake:

> *Pork meat, eaten two times a week, two pieces each time / Pork meat-serving size is one piece, 25 g.*
> *Amount of pork meat consumed per day = 0.29 x 2 x 25 g = 14.28 g*

2.6. Statistical analysis

All food groups, food items, energy and food nutrients were coded and the descriptive statistics were calculated (i.e. mean value, standard deviation, median and 95th percentile) for both individual and total consumption. Data entering and coding are long and repetitive operations and they represent a crucial step of the study. Daily consumptions (expressed in gram of food per person per day) and the value of dietary energy or nutrient intake (expressed in mg per person per day) were reported as mean value ± standard deviation. Non-consumers of food group were excluded from the estimation, and the percentage of consumer was also calculated. Gender, seasonal and regional differences were calculated using Independent-Sample T-test in order to consider a significant difference for the data following normal distributions, and Kruskal-Wallis test for non-parametric data. The mean difference (difference observed) was recorded and a p-value < 0.05 was considered significant. All data analysis was performed using SPSS version 19.0(SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Socio-demographic characteristics of studied population

The 941 study participants were recruited in the four regions of Cambodia with 441 (46.9%) people during the wet season and 500 (53.1%) people during the dry season. The number of subjects was almost similar in the four regions: with 25% in R1, 25% in R2, 31% in R3 and 19% in R4 (**Table 3.2**). Gender distribution was 36% men and 64% women. The average age was 39.6±12.2 and 40.5±11.6 years for men and women, respectively. The predominant age class was between 25 and 34 year old, representing 40%, followed by 22% for the age class between 35-44 years, 23% for the age class of 45-54 years, and the minor group was the last age class between 55-65 year old, with 15%. A high proportion of participants had a low education level, with primary school and illiterate representing almost 45% of the total participants. There are differences between genders in term of occupational status and educational level: notably, the proportion of men and women holding professional jobs was 37 and 11% respectively; the percentage of men and women at university was 20 and 7.8%respectively.

Participants had no serious diseases such as cancer or inflammatory bowel disease. In our population, 1.06% of participants were diabetic. Results showed that near 14% of participants were smokers, most of those being men (87%). Quantitatively, 85% of smokers smoked around one pack per day. Most of the physical activities of the Cambodian population were related to their daily activity; almost none of participants in the study group went to the sport centre.

The average of body mass index (BMI) of the studied population is 20±3.5kg/m^2. In this study, overweight (defined by BMI in the range 25-29.9kg/m^2) and obesity (BMI ≥ 30kg/m^2) were found in 14 and 2.4% of the population, respectively. Malnutrition was also noticed in the studied population: 12% of subjects had a BMI < 18.5kg/m^2, and 82% of these were women.

Table 3.2. Demographic and socio-economic data of the study.

	Number of participants	Percentage (%)
Participants		
Region 1	238	25.3
Region 2	233	24.8
Region 3	289	30.7
Region 4	181	19.2
Women	603	64.1
Men	338	35.9
Age distribution		
25-34 years old	378	40
35-44 years old	207	22
45-54 years old	216	23
55-65 years old	140	15
Education		
University	115	12.2
High school	161	17.1
Secondary school	238	25.3
Primary school	318	33.8
No study	109	11.6
Occupation		
Student	42	4.5
Employee and official (civil servant)	175	18.6
Own account worker and employed unpaid	299	31.8
Housewife	200	21.2
Farmer	207	22.0
Not working	18	1.9
Smoking		
Not smoking	810	86.1
Smoking	131	13.9
Alcohol consumption		
Regularly	63	15.1
Often	116	27.8
Occasionally	160	38.4
Rarely	78	18.7
Never	524	55.7
Body Mass Index (kg/m^2)		
<18.5	115	12.2
18.5–24.9	672	71.4
25–29.9	131	13.9
≥ 30	23	2.4
Physical activity		
Walking	858	91.2
Bicycle	226	24.0
Working in field/garden	231	24.5
Household task	752	79.9
Sport centre	4	0.4

3.2. Food consumption patterns

3.2.1. Characteristics including all recorded data

For all participants, the mean daily consumptions per food item for both seasons and both sexes were calculated, as well as the consumption at the 95th percentile of the distribution. The results are presented in **Table 3.3** for the 14 food groups, together with the percentage of consumers.

Table 3.3. Level of consumers and food consumption of 14 food groups.

Food groups	**Food code**	**Consumers (%)***	**Food consumption (g/person/day)**	
			Mean ± SD	95th percentile
Cereals and products	A			
Rice		100	823±334	1425
Other cereals and products		72.2	110±77	250
Starchy roots, tubers	B	23.7	32±53	109
Legumes, nuts, seeds	C	31.5	38.5±42	114
Vegetables and products	D	98.5	250±101	421
Fruits and products	E	55.8	145±125	400
Meat and products	F	96.8	75±40	144
Fish, shellfish and products	G	96.8	75±37	141
Eggs and products	H	36.7	35±25	85
Milk and milk products	J	21	67±49	170
Fats, oil and products**	K	89.8	23±18	55
Sugar and confectionary**	M	90.8	22.7±20	62
Condiments and spices**	N	95.4	42±22	82
Beverage, alcoholic	P	5.6	255±164	548
Amount of alcohol		5.6	26±16	60
Beverage, non-alcoholic	Q	54	261±201	683

**: Total consumption for both seasons and both sexes*

***: Include the specific study on ingredient consumption*

All participants (100%) in this study consumed rice at least twice a day, which is representative of the Cambodian population. The daily consumption of cooked rice was 823 g/person/day, the value increasing to 1,425 g/person/day for high consumers. The mean consumption of other cereals was 110 g/person/day. Corn, which is the second highest crop production in Cambodia, was found as

frequently consumed by studied population; on the opposite, at all the study sites, very small amount of wheat was consumed.

The other food groups consumed by more than 95% of the adult Cambodian population considered in this study were vegetables, fish and meat. Percentage of fruit consumers was 56%. On average, the studied population consumed 250 g/person/day of vegetables and 145 g/person/day of fruits, with high consumers ingesting 421 and 400 g/person/day respectively of vegetables and fruits. On average, this population consumed about 75 g/person/day of meat and the same quantity of fish, with high consumers ingesting 144 and 141 g/person/day respectively of meat and fish.

The results of this survey show that the Cambodian diet mainly consists of cereals, fish, meat, fruits and vegetables. Nuts and seeds, starchy roots, tuber and products were also found in minor amounts in the diet. The mean daily consumption of nuts, seeds and their products was 38.5 g/person/day. The main soybean food items consumed in Cambodia are fermented soy bean, soy bean curd, soy noodle, and soy milk. The results showed that the most consumed starchy roots, tuber, and their products in Cambodia are sweet potato, taro tuber, tapioca, tapioca flour, and tapioca granule.

The average consumption of milk was 67 g/person/day (61 ml/person/day) of the studied population, which is in agreement with the fact that milk and dairy products are not widely use in Cambodia. The mean consumption for eggs was 35g/person/day, duck eggs being the most consumed.

The common cooking methods were boiling, stewing, grilling, backing and frying, with Cambodian cooking habit being salty and sweet taste. Historically, herbs and spices have enjoyed a rich tradition of use for the flavor enhancement characteristics and for their medical properties. Fresh spices and herbs were common, basic ingredients in most traditional Cambodian cooking. Overall, the Cambodian population in this study consumes in average about 23 g of fats and oils, 21 g of sugars and 42 g of condiment and spices per person per day.

Non-alcoholic beverages including tea, coffee, and soft drinks were consumed by 54% of the studied population. The results showed that tea is the favorite and most frequently consumed beverage in both urban and rural areas. In contrast, soft drinks are not as frequently consumed in the rural areas due to their high price. The percentage of consumers of alcoholic beverages is 5.6%. On average, adult Cambodians in this study consume 255 g/person/day of alcoholic beverages, corresponding to around 26 g of alcohol per person per day. The consumption for high consumers of alcoholic beverages is about 650 g/person/day, corresponding to 60 g of alcohol per person per day.

3.2.2. Differences observed between genders

The mean consumptions of men and women in both seasons for the 14 food groups and the p-value are presented in **Table 3.4**.

Table 3.4. Difference in food consumption between men and women in Cambodian population.

Food groups	**Consumers (%)**		**Mean ± SD** (g/person/day)		**Mean-dif**[1]
	Men	Women	Men	Women	
Cereals and products					
Rice	100	100	1021.8±345.2	711.7 ± 271	310.1**
Other cereals and products	66.6	75.3	116.8±85	106.1 ± 73	10.7
Starchy roots, tubers	21.6	24.9	33.3±50.5	31.7 ± 54.1	1.6
Legumes, nuts, seeds	27.5	33.7	38.7±43.1	38.4 ± 41.8	0.3
Vegetables and products	98.5	98.5	257.3±106.9	245.7 ± 97.7	11.6
Fruits and products	48.5	59.9	138.4±137.9	147.8 ± 119	-9.4
Meat and products	95.9	97.3	80.3 ± 42.8	71.7 ± 38.5	8.6*
Fish, shellfish and products	97.3	96.5	79.8 ± 39.6	72± 35.2	7.8*
Eggs and products	32.2	39.1	39.1 ± 29	32.8± 23.1	6.3*
Milk and milk products	22.2	20.4	73.8 ± 45.9	63 ± 51	10.8
Fats, oil and products	82.5	93.9	20.8 ± 18	23.5 ± 17.2	-2.7*
Sugar and confectionary	79.9	96.8	20.8 ± 18	23.6 ± 20.8	-2.8
Condiments and spices	90.2	98.3	37.2 ± 23.7	44.5 ± 20.4	-7.3*
Beverage, alcoholic	10.4	3	294 ± 180	178.6 ± 92.1	115.4*
Quantity, alcohol	10.4	3	25.4±13.6	27.9±20	-2.5
Beverage, non-alcoholic	63	48.9	301± 222	231.7 ± 178	69.3**

Consumption values are mean ± standard deviation (95% confidence interval).

Independent-Sample T-test performed at p-value<0.05, between men and women (p-value < 0.05; ** p-value <0.01).*

[1]: Mean-dif: Mean difference.

Significant differences in consumption were observed between men and women for rice; meat and their products; fish, sea food and their products; eggs; fats and oil; condiments and spices; alcoholic and non alcoholic beverages. The mean consumption of these food groups, except condiments and spices, was significantly higher for men than for women ($p < 0.05$). The rice consumption was

found to be very high for men, with an average daily consumption of 1021.8 g/day, compared to 711.7 g/day for women. Similarly the 95th percentile of consumption was higher for men than for women, with respectively 1605 vs. 1200 g/day. The consumption of alcoholic and non-alcoholic beverages were significantly higher for men than women, and the percentage of consumers was also higher for men than women, with 10 and 3% for alcoholic beverages, and 63 and 49% of non-alcoholic beverages respectively. However, the amount of alcohol consumed (in g per person per day) was found to be not significantly different between men and women. The daily consumption of fruits and vegetables also did not differ significantly between men and women.

3.2.3. Differences observed between seasons and regions

Differences in mean consumption between the wet and dry seasons are described in **Table 3.5**. The consumption of rice, vegetable, starchy roots and tubers, as well as condiments and spices were significantly higher in the wet season ($p<0.05$). On the contrary, the consumption of alcoholic and non-alcoholic beverages was higher in the dry season, but without significant difference for alcohol consumption. The daily consumption of other food groups was not significantly different between the wet and the dry seasons.

The mean consumption by region and the regional variations of dietary consumption in the four different regions in Cambodia are presented in **Table 3.6 and Table 3.7**. The daily consumption of rice differs significantly between R1-R2, R1-R4, R2-R4 and R3-R4 ($p<0.05$). The lowest rice consumption was observed in R4 with a mean consumption of 743 g/person/day. The highest rice consumption was observed in R2 with 880 and 1,467 g/person/day for the mean and 95th percentile respectively. Daily consumption of vegetable was similar in R1, R2 and R3 with about 260 g/person/day, but it is significantly lower in R4 (222 g/person/day). The daily consumption of fruits differed significantly between R1-R2 and R1-R4 ($p<0.05$).

The consumption values for meat, fish and their products were significantly different between regions. In the capital (R1), consumers consumed significantly more meat (93 and 158 g/person/day respectively for mean and 95th percentile) compared with the other regions. However, fish and seafood were more consumed in R4 (82 and 163 g/person/day respectively for mean and 95th percentile) compared to the other regions. Significantly different egg consumptions were found between R1-R2 and R2-R3. The other cereals, starchy roots and tubers, legumes and nut, and alcoholic beverages did not differ between regions, except other cereals and their products that were

found to differ significantly between R1-R2. Fats, oil and sugar consumption were significantly different between R1-R2, R2-R3 and R2-R4. Significantly different consumption of condiments and spices were found between R1-R3, R1-R4, R2-R3 and R2-R4. The non-alcoholic beverage consumptions were significantly different between R1-R3 (p=0.02), R2-R3 (p=0.01), R3-R4 (p=0.001).

Table 3.5. Seasonal variations of food consumption in Cambodia (in g/person/day).

Food groups	**Consumers (%)**		**Mean ± SD**	
	Wet season	Dry season	Wet season	Dry season
Cereals and products				
Rice	100	100	880.8±349.5	772.3±312.2**
Other cereals and products	75.3	69.4	109.6±75.2	109.7±78.8
Starchy roots, tubers	23.1	24.2	40.7±70.8	25.1±28.8*
Legumes, nuts, seeds	31.7	31.2	37.8±48.6	39.2±35.5
Vegetables and products	99.3	97.8	262.6±106.7	238.4±94.7**
Fruits and products	55.1	56.4	146.3±138.1	143.5±113.2
Meat and products	98	95.8	77.2±39.5	72.68±40.9
Fish, shellfish and products	96.8	96.8	72.4±37.4	76.96±36.6
Eggs and products	17.7	35.6	36.8±24.9	33±25.4
Milk and milk products	16.8	24.8	59.4±46.9	71.6±50
Fats, oil and products	93.4	86.6	22.4±15.8	22.8±19
Sugar and confectionary	93	88.8	23.8±21.4	21.8±18.1
Condiments and spices	98	93.2	44±21.3	40.3±22.1*
Beverage, alcoholic	3.4	7.6	189.5±121.6	280.7±173.1*
Quantity, alcohol	3.4	7.6	21.6±15.3	28.1±15.9
Beverage, non-alcoholic	47.8	59.4	221.8±180.3	288.4±210**

Consumption values are mean ± standard deviation (95% confidence interval).

Independent-Sample T-test performed at p-value <0.05 between wet season and dry season (p-value < 0.05;** p-value <0.01).*

Table 3.6. Regional variations of food consumption in Cambodia, expressed as mean±SD (in g/person/day).

Food groups	**R1**	**R2**	**R3**	**R4**
Cereals and products				
Rice	817.5±315.8	880.4±326.5	831.8±333	743±356
Other cereals and products	98.5±66.5	122.2±79.5	147.9±84.7	114.2±74.1
Starchy roots, tuber and products	23.1±29.7	45.2±73	28.5±35.8	30.9±68
Legumes, nuts, seeds	37.9±39.7	38.4±34.1	33.9±41.4	48.4±53.9
Vegetables and products	259.9±107.7	251.5±92.1	257.9±105.6	221.6±90.4
Fruits and products	169.4±141.4	131.8±104.4	140.6±126.1	125.4±110.9
Meat and products	93.1±38.6	62.6±37.5	69±41.9	74.8±36.5
Fish, shellfish and products	65.4±37.8	76.2±34.2	76.9±36.4	82.1±38.5
Eggs and products	39.7±30.2	28.5±17.7	36.5±27.5	33.5±19.5
Milk and milk products	70±48.5	61.1±34.8	72.6±56.9	63.5±57.1
Fats, oil and products	22.5±15.8	26.1±21	21.8±15	19.2±18.9
Sugar and confectionary	21.3±14.8	28±27	21.1±19.4	20.8±16.5
Condiments and spices	46.6±20.8	44.7±27.6	38.8±18.4	37.6±18.2
Beverage, alcoholic	247.2±192.3	262.2±192.6	261±153.7	246±163.5
Quantity, alcohol	15.4±10.9	29.9±19.9	27.1±18	28.6±10.3
Beverage, non-alcoholic	253.3±200.2	289.4±234.8	205.1±149.5	294.9±195

Table 3.7. p-Values for differences in consumption of 14 food groups in four regions in Cambodia.

Food groups	**p-Values***					
	R1-R2	R1-R3	R1-R4	R2-R3	R2-R4	R3-R4
Cereals and products						
Rice	0.03	0.61	0.02	0.1	0.001	0.006
Other cereals and products	0.004	0.36	0.06	0.05	0.37	0.34
Starchy roots, tubers	0.07	0.4	0.5	0.07	0.36	0.8
Legumes, nuts, seeds	0.93	0.54	0.22	0.45	0.21	0.06
Vegetables and products	0.37	0.84	0.002	0.47	0.001	0.001
Fruits and products	0.02	0.05	0.01	0.54	0.68	0.34
Meat and products	0.001	0.001	0.001	0.07	0.001	0.13
Fish, shellfish and products	0.01	0.01	0.001	0.83	0.11	0.15
Egg and products	0.003	0.45	0.17	0.02	0.12	0.45
Milk and milk products	0.27	0.8	0.54	0.22	0.8	0.47
Fats, oil and products	0.04	0.64	0.07	0.01	0.02	0.12
Sugar and confectionary	0.001	0.9	0.75	0.002	0.003	0.86
Condiments and spices	0.4	0.001	0.001	0.04	0.004	0.5
Beverage, alcoholic	0.87	0.84	0.98	0.98	0.83	0.78
Quantity, alcohol	0.08	0.09	0.009	0.71	0.83	0.78
Beverage, non-alcoholic	0.17	0.02	0.09	0.01	0.84	0.001

**: Independent-Sample T-test performed between regions at p-Value < 0.05*

3.2.4. Difference observed between age-ranges

The mean consumption by age range of dietary consumption in Cambodia is presented in **Table 3.8**. The daily consumption of rice differs significantly between the four age ranges ($p<0.05$). The highest consumption was in the first age range; follow by the second age range (760 vs 894 g/person/day). Meat intake was also found highest in the first age range and lowest in the last age range (58 vs 86 g/person/day) ($p<0.05$). In contrast, mean intake of fish was highest in the last age range and lowest in the first age range (71 vs 84 g/person/day, $p<0.05$). Significant differences in consumption were observed between age ranges for egg, milk and milk products. The daily consumption of other food groups was not significantly different between the wet and the dry seasons.

Table 3.8. Variations of food consumption in Cambodia by age, expressed as mean±SD (in g/person/day).

Food groups	25-34	35-44	45-54	55-65	p-Value
Cereals and products					
Rice	894±355	819±284	744±317	760±335	<0.05
Other cereals and products	105±71	116±76	110±80	111±87	>0.05
Starchy roots, tubers	36.6±63	26±30	28±33.5	33.6±64.6	>0.05
Legumes, nuts, seeds	38±41	44±49	37±40	36±38	>0.05
Vegetables and products	254±103	245±101	245±100	254±100	>0.05
Fruits and products	145±126	151±119	139±126	145±133	>0.05
Meat and products	86.2±40	70.6±40.3	69.4±36.4	58±38.6	0.001
Fish, shellfish and products	71±38	74±36	76±36	84±36	<0.05*
Egg and products	40±29	30±20	29±19	32±23	<0.05
Milk and milk products	60±50	57±46	59±38	67±45	<0.05
Fats, oil and products	23±19	23±17	22±16	23±18	>0.05
Sugar and confectionary	25±24	21±16	20±13**	24±21	>0.05
Condiments and spices	42±22	40±24	42±19	46±22	>0.05
Beverage, alcoholic	284±187	250±148	249±168	179±95	>0.05
Quantity, alcohol	23±29	26±10	29±24	24±4	>0.05
Beverage, non-alcoholic	249±186	239±194	292±219	271±213	>0.05

**: The last age range compare to the other age ranges; **: p<0.05: between the first and the third range.*

3.2.5. Cooking practices

Table 3.9 shows the detailed mean consumptions of meat, poultry, fish, sausage, and offal products (g/person/day) for men and women, wet and dry seasons, according to cooking method in Cambodia, obtained from FFQ.

Most participants preferred to consume red meat (pork and beef), poultry, fish, sausage and offal products after grilling and pan-frying. It can be noted that the studied population consumed more pork than other meats. We did not found any significant difference in intake of most products according to cooking methods between men and women, wet and dry seasons. One exception was fish consumption after grilling, being significantly different between men and women (p=0.007) in

both seasons (p=0.001). Also, sausage consumption was significantly different in both seasons when grilled (p=0.002) or pan-fried (p=0.03).

Table 3.9. Consumptions of specific foods in studied population, according to cooking practices in Cambodia.

Food items	Mean±SD (in g/person/day)			
	Men	**Women**	**Wet season**	**Dry season**
Grilled				
Pork	13±18	12±13	12±14	12±17
Beef	11±14	10±15	11±16	9±12
Poultry	8±13	6±10	7±13	6±9
Fish	28±27	23±22*	21±19	28±28*
Sausage	6±8	5±8	7±10	4±6**
Offal products	12±22	6±8*	10±18	4±3
Pan-fried				
Pork	14±18	13±15	14±15	14±17
Beef	8±11	11±16	11±13	9±15
Poultry	9±23	6±9	8±12	7±18
Fish	23±22	21±21	21±19	22±23
Sausage	4±5	5±7	5±8	4±4
Offal products	7±10	9±8	10±12	6±6
Deep-fried				
Poultry	10±18	7±9	10±16	6±9
Fish	12±13	11±16	9±11	13±17
Baked				
Poultry	6±14	7±11	8±15	5±9
Fish	5±7	8±12	8±12	4±5

Consumption values are mean ± standard deviation (95% confidence interval)

Kruskal-Wallis test for non-parametric data performed at p-value<0.05 between men and women; and wet season and dry season (p-value < 0.05; ** p-value<0.01).*

3.3. Energy and nutrient intake

3.3.1. Energy and macronutrient intake

Table 3.10 presents the distribution of energy, protein, fat, carbohydrate and dietary fiber intake of studied population. The mean daily energy intake of men (2097.5 kcal/day or 86.1% of recommended nutrient intakes (RNIs)) was higher than that of women (1731.4 kcal/day or 89.4% of RNIs). In generally, energy intake achievement in both sexes was less than the value of the recommended dietary allowances (RDA) for Southeast Asia (total energy intake: 2436 kcal/day for men and 1937 kcal/day for women) and declined gradually with age. Differences were observed between seasons, with, as expected, a higher daily energy intake during the wet seasons. Surprisingly, even though differences between regions could be noted (the highest energy intake being observed in the capital (1914.7 kcal) and R2 (1925 kcal), and the lowest in R4 (1748.3 kcal), they were not statistically significant. Carbohydrate, protein, fat and dietary fiber intake declined as the persons become older. Rice contributed to about 53% of the total daily energy intake.
Meat and fish provided 9.1 and 6.8% of the total daily energy intake, respectively. Cambodian adults recorded a mean protein daily intake of 65.9 g, which represents 13.7% of the total daily energy intake. The mean protein intake of men was 73.2 g per person, significantly ($p \leq 0.01$) higher when compared to women (61.9 g). The mean consumption of protein was similar in both seasons (near 66 g) and in all studied regions.

The average contribution of fats and sugar was 6.4 and 2.8%, respectively. Mean daily intake of fat was estimated to be about 37.6 g for the whole studied population, which represents 17.5% of total daily energy intake. A similarity in fat intake was noted between both sexes, as well as between regions (the highest intake was found in R1 (42.8 g) and the lowest in R3 (36.1 g), with statistical significance of this difference, $p<0.05$). The mean carbohydrate intake of Cambodian population in this study was approximately 331.9 g, which contributed to 68.8% of the total daily energy intake. The mean carbohydrate daily intake was highest in R2 (349.2 g) and lowest in R4 (313 g), but this was not statistically significant ($p>0.05$). On the opposite, the difference of mean carbohydrates consumption was statistically significant between genders (being 392.5 and 298 g per person for men and women, respectively) as well as between seasons ($p<0.05$). The mean consumption of vegetables and fruits reached the international recommendations, and contributed to 3.9 and 5.8% of the total daily energy intake, respectively. The mean dietary fiber intake was estimated to be 12.5 g per person in the studied population and not different between men and women.

Table 3.10. Energy and macronutrient intake of studied Cambodian adult population (kcal, g/person/day).

Characteristics of the studied population	Energy (kcal)		Protein (g)		Fat (g)		Carbohydrates (g)		Fiber (g)	
	Mean±SD	95th	Mean±SD	95th	Mean±SD	95th	Mean±SD	95th	Mean±SD	95th
All study (N =941)	1862.9±514.6	2750.9	65.9±17.2	94.5	37.6±14.6	63.8	331.9±108.4	511.9	12.4±4.8	22.1
Men (N=338)[1]	2097.5±528.1**	2953.5	73.2±17.1**	99.6	39.2±39.1	64.9	392.5±109.3**	574.4	13.3±5.2**	24.2
Women (N= 603)[1]	1731.4±457.4**	2518.3	61.9±15.9**	88.2	37.2±14.4	61.7	298±92**	450.9	11.9±4.6**	21.2
Seasons										
Wet season (N= 441)[2]	1934±525.5**	2825.1	65.9±16.7	93	38.4±14.2	64.6	347.4±111.9**	523.1	13.2±5.2**	23.3
Dry season (N =500)[2]	1800.1±497**	2686.3	66±17.6	95.5	36.8±14.9	63.1	318.2±103.3**	500.6	11.8±4.4**	20.8
Regions[3]										
Region 1 (N=238)	1914.7±508.4	2753	67.6±16.5	94.6	42.8±14.5	72.6	329.3±107.6	505.2	12.7±5	22.2
Region 2 (N=233)	1925.2±525.5	2812.2	64.6±16.4*	93.1	37.1±16.5**	65.9	349.2±111.3	509.7	13±5.2	25.2
Region 3 (N=289)	1841.7±499.8	2699.6	65.9±17.8	94.5	36.1±12.2**	58.2	331.7±103.8	514.4	12.6±4.6	21.6
Region 4 (N=181)	1748.3±514.4**	2740.8	65.5±18	96.6	37.1±13.8**	57.6	313.2±110.2	522.9	11.1±4.4**	19.4
Age-class[4]										
25-34 years (N=378)	1974.7±508.1	2873.3	68.7±16.8	95.6	39.1±15.4	65.9	354.4±108.8	534.1	13.1±5.1	22.9
35-44 years (N =207)	1851.4±462.4*	2686.7	65±16.4*	92.3	37.5±14.8	64.3	331.6±97.1*	501.3	12.4±4.6	21.7
45-54 years (N =216)	1749.3±489.6**	2581.5	63.1±16.3**	89.5	36.7±13.3*	60.5	306.5±104.8**	480.4	11.5±4.6*	20.7
55-65 years (N=140)	1753.1±580.4**	2725.4	64.2±19.6**	98.6	34.9±13.3*	63.7	310.4±116.3**	518.4	11.8±4.8*	22.2

Independent-Sample T-test performed at p-value <0.05 between wet season and dry season (p-value < 0.05; **p-value <0.01).*

[1]: Independent-Sample T-test performed between men and women; [2]: Independent-Sample T-test performed between wet season and dry season
[3]: Independent-Sample T-test performed between regions (compared to the region 1); [4]: Independent-Sample T-test performed between age ranges (performed between the first age range with the other age ranges).

The food contribution patterns for macronutrients differed between the elderly and younger adults in the following ways as presented in Table 3.8. The total of energy intake was highest in the first age range and the lowest in elderly group in the study populations (1974.9 vs 1753 kcal/person/day). The energy intake was found with a difference significantly as compared to the first age range ($P<0.05$). The highest proportion of the three macronutrients was in the younger adults groups of study population. A difference of the intake of these three macronutrients was high noticed between the first the third and last age group ($p <0.05$) as presented in Table 3.10.

3.3.2. Mineral intake

Diets rich in several minerals may modulate risk of CRC. For example, dietary calcium may bind with the bile acids in the bowel lumen, inhibiting their proliferative and carcinogenic effects. In contrast, red meat and processed rich in heme-iron seem to increase CRC risk. In this part, we focus on several minerals (calcium, iron and sodium) exposure assessment of the Cambodian population. **Table 3.11** presents the mean intake and 95th percentile of several minerals for the studied Cambodian population.

Cambodian adults recorded a mean daily intake of about 609.5 mg for calcium, with dry season's adults having a higher intake at wet season. Percentage of RNI achievement for calcium was 64% for adults in both sex and all age ranges. Calcium consumption was lowest for the youngest age group and highest in the last age range in both sexes (591 vs 666 mg/person/day, $p<0.05$). By region, mean consumption of calcium ranged from 572 to 661 mg/person/day in R1 and R3, respectively, but differences were not statistically significant.

The average intake of iron among the studied population was 12.5 mg/person/day for adults, with significant ($p <0.01$) differences noted between men and women (13.3 and 12 mg/person/day, respectively). By age group, season and all regions, RNI achievement was lower in women (44% of RNI) than in men (72.7% of RNI). Iron consumption was found similar in all regions. The mean intake of iron was higher in the first age range (13 mg/person/day) and similar for the other three age ranges (12 mg/person/day) but not difference significantly between groups ($p>0.05$).

The average mean of sodium intake was 2996 mg for adult Cambodian population in this study. Cambodian women consumed about 299 mg of sodium more than men, while the intake was higher in wet season compared to dry season. The highest intake of sodium was in the last age range while the other three-age ranges had similar intakes (2919 vs 3301 mg/person/day). By region, sodium

consumption ranged from 2684 to 3185 mg/person/day in R1 and R4 respectively, being not statistically different between regions ($p>0.05$), except region one and four with a statistically difference ($p<0.05$).

Table 3.11. Mineral intake of studied Cambodian adult population (in mg/person/day).

Characteristics of studied population	Calcium		Iron		Sodium	
	Mean±SD	95th	Mean±SD	95th	Mean±SD	95th
All study (N =941)	609.5±304	1159.6	12.5±4.2	19.7	2996±1799	6287.6
Men (N=338)[1]	661.3±298**	1202.9	13.3±4**	20.4	2805±1687*	5622.4
Women (N= 603)[1]	580.5±303**	1132.6	12±4.2**	19.2	3104±1852*	6692
Seasons						
Wet season (N= 441)[2]	554.9±266**	984.6	12.7±4.3	20.7	3118±1778*	6680.4
Dry season (N =500)[2]	657.7±327**	1247.5	12.3±4	19.4	2889±1813*	6265.8
Regions[3]						
Region 1 (N=238)	572±286.5	1072.1	12.5±3.9	19.6	3185±2103	7682.1
Region 2 (N=233)	592±275.6	1066.2	12.4±4.3	20.1	3076±1837	6273
Region 3 (N=289)	661±319.8**	1242.7	12.7±4	19.7	2973±1712	6279
Region 4 (N=181)	598±325	1245.2	12.2±4.4	20.3	2684±1373*	5354
Age-class[4]						
25-34 years (N=378)	591±291.7	1068.2	13±4.3	20.3	2919±1935	6572.3
35-44 years (N =207)	591.6±288	1087	12.2±3.7*	19.3	2947±1753	6104.3
45-54 years (N =216)	622.3±317	1224.7	12.1±4.1	19.1	2981±1528	6250
55-65 years (N =140)	666.4±333**	1329.4	12.2±4.4	19.5	3301±1859*	7041

Independent-Sample T-test performed at p-value <0.05 between wet season and dry season (p-value < 0.05; **p-value <0.01).*

[1]: Independent-Sample T-test performed between men and women

[2]: Independent-Sample T-test performed between wet season and dry season

[3]: Independent-Sample T-test performed between regions (compared to the region 1);

[4]: Independent-Sample T-test performed between age ranges (performed between the first age range with the other age ranges).

3.3.3. Vitamin intake

Nutrition and health are closely connected and malnutrition can seriously endanger health; the consequences are higher risks of developing diseases. Vitamins are important micronutrients that can

affect the health of the population. Most studies of diet and chronic disease such as cancer have considered the role of nutrients and micronutrients, such as fat, fibers, mineral and vitamins (folate, beta-carotenoid, vitamin C, etc). We here focused on vitamins A, B1, B2, C and niacin intake for the studied population.

Vitamin A intake in the studied population was about 0.41 mg/person/day (see **Table 3.12**), which represented about 74% of RNI. By gender, vitamin A intake for men and women was 72.2 and 73.7% of RNI, respectively. In term of regional variations, vitamin A intake was the highest (0.45 mg/person/day) in the capital and the lowest in R2 (0.35 mg/person/day), even though differences were not statistically significant ($p>0.05$). Vitamin A consumption was quite similar at all age ranges.

The mean intake of thiamin (vitamin B1) of the respondents was 0.68 mg/person/day. Men (58.3% of RNI) and women (54.5% of RNI) had similar achievements of RNI across ages, seasons and regions. The average consumption of riboflavin (vitamin B2) was 0.86 mg/person/day for adult population in this study, while intake of both men and women was about 69 and 72% of RNI, respectively. Similar intakes of riboflavin were seen in all regions, as well as within the age-classes.

The intake of niacin was higher in men (14.7 mg/person/day, 91.8% of RNI) than in women (12.2 mg/person/day, 87.1% of RIN). Among regions, no significant differences could be observed despite slightly higher niacin consumption in the capital (13.6 mg/person/day). A progressive but not significant decrease in niacin intake was noted with age ($p>0.05$). A significant difference in niacin consumption was found between wet and dry seasons ($p <0.05$).

The mean intake of vitamin C of the respondents was about 70.9 mg/person/day, with women's intake (68 mg/person/day) being slightly higher than that of men (72 mg/person/day), even though not significant. In this study, the Cambodian adult intake vitamin C reached its recommended value (100% of RNI). Vitamin C intake was found to be higher in wet season than dry season. Overall, vitamin C intakes were similar among regions and age-classes (despite slightly higher values in the capital and for the 25-34 years).

Table 3.12. Vitamins intake of Cambodian adult population (in mg/person/day).

Characteristics of the studied population	Vitamin A		Vitamin B1		Vitamin B2		Niacin		Vitamin C	
	Mean±SD	95th	Mean±SD	95th	Mean±SD	95th	Mean±SD	95th	Mean±SD	95th
All study (N =941)	0.41±0.34	1.07	0.65±0.2	1.06	0.86±0.3	1.4	13.1±4	19.7	70.9±58	175.2
Men (N=338)[1]	0.43±0.38	1.2	0.7±0.2**	1.14	0.9±0.3**	1.4	14.7±4**	20.7	68.4±58	156.3
Women (N= 603)[1]	0.39±0.32	1	0.6±0.2**	1.02	0.8±0.27**	1.3	12.2±3.7**	17.9	72.2±58.9	180.3
Seasons										
Wet season (N= 441)[2]	0.39±0.32	0.99	0.7±0.2	1.03	0.86±0.26	1.3	13.7±4.2**	20.3	77.4±72*	228
Dry season (N =500)[2]	0.4±0.36	1.17	0.6±0.2	1.07	0.86±0.29	1.4	12.6±3.7**	19	65±41.1*	146
Regions[3]										
Region 1 (N=238)	0.44±0.32	1.04	0.68±0.2	1.1	0.9±0.3	1.4	13.6±3.8	20.4	83.7±62.3	200.7
Region 2 (N=233)	0.35±0.3	1.04	0.65±0.2	1.1	0.85±0.3	1.3	13.1±3.7	18.9	67.5±68.4	165.6
Region 3 (N=289)	0.44±0.38	1.3	0.6±0.2	1.0	0.8±0.3	1.2	13.1±4.2	19.8	67±48.6**	171.1
Region 4 (N=181)	0.37±0.33	1.08	0.65±0.2	1.0	0.86±0.3	1.4	12.4±4	19.5	63.9±48**	154.4
Age-class[4]										
25-34 years (N=378)	0.4±0.36	1.0	0.7±0.2	1.08	0.9±0.27	1.4	14±3.8	20.4	75.9±61.1	195.06
35-44 years (N =207)	0.4±0.37	1.2	0.64±0.2	1.04	0.83±0.25	1.3	12.7±3.4	18.7	68.5±51	168.9
45-54 years (N =216)	0.38±0.3	0.9	0.6±0.2	1.03	0.83±0.27	1.3	12.3±3.6	18.3	66.7±55.1	158.4
55-65 years (N =140)	0.42±0.32	1.08	0.6±0.3	1.1	0.83±0.3	1.4	12.4±5.1	20.9	67.1±62.7	200.6

Independent-Sample T-test performed at p-value <0.05 between wet season and dry season (p-value < 0.05; **p-value <0.01).*

[1]: Independent-Sample T-test performed between men and women; [2]: Independent-Sample T-test performed between wet season and dry season.
[3]: Independent-Sample T-test performed between regions (compared to the region 1); [4]: Independent-Sample T-test performed between age ranges (performed between the first age range with the other age ranges).

3.4. Food contributor to energy, macro nutrients and micronutrients

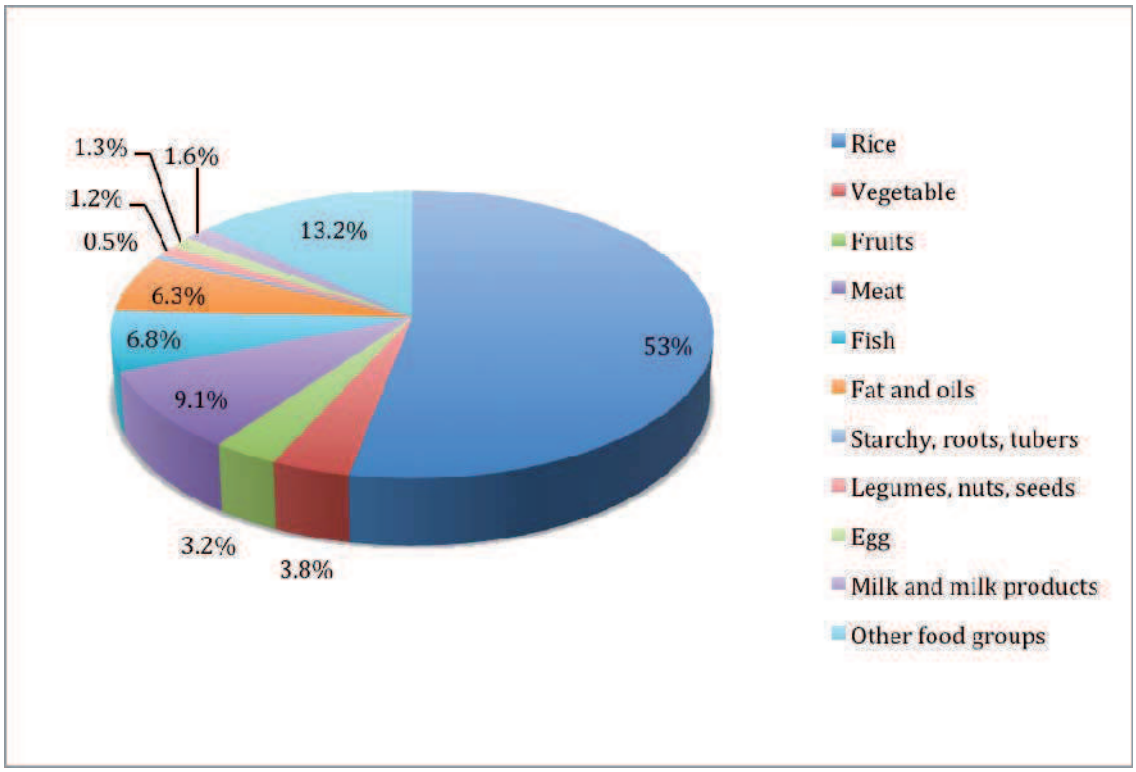


Figure 3.3. The major food groups contributed to the total energy intake of Cambodian population

Figure 3.3 shows a major food contributor to the total energy intake amount 941 studied populations in Cambodia. The cereal group provided the most of the total energy intake and in which rice contributed 53%. The second and the third food groups were meat and fish (9.1% and 6.8%, respectively).

Figure 3.4, 3.5, 3.6 and 3.6 show the principle food groups contributed to total protein, fat, carbohydrate and dietary fiber intake of the studied population. Data for mean intake presented in table Table 3.10 with the mean intake of protein was 65.9 g/person/day in which fish (29%) was the main food contributor of protein intake follow by rice (27.4%) and meat (24.4%).

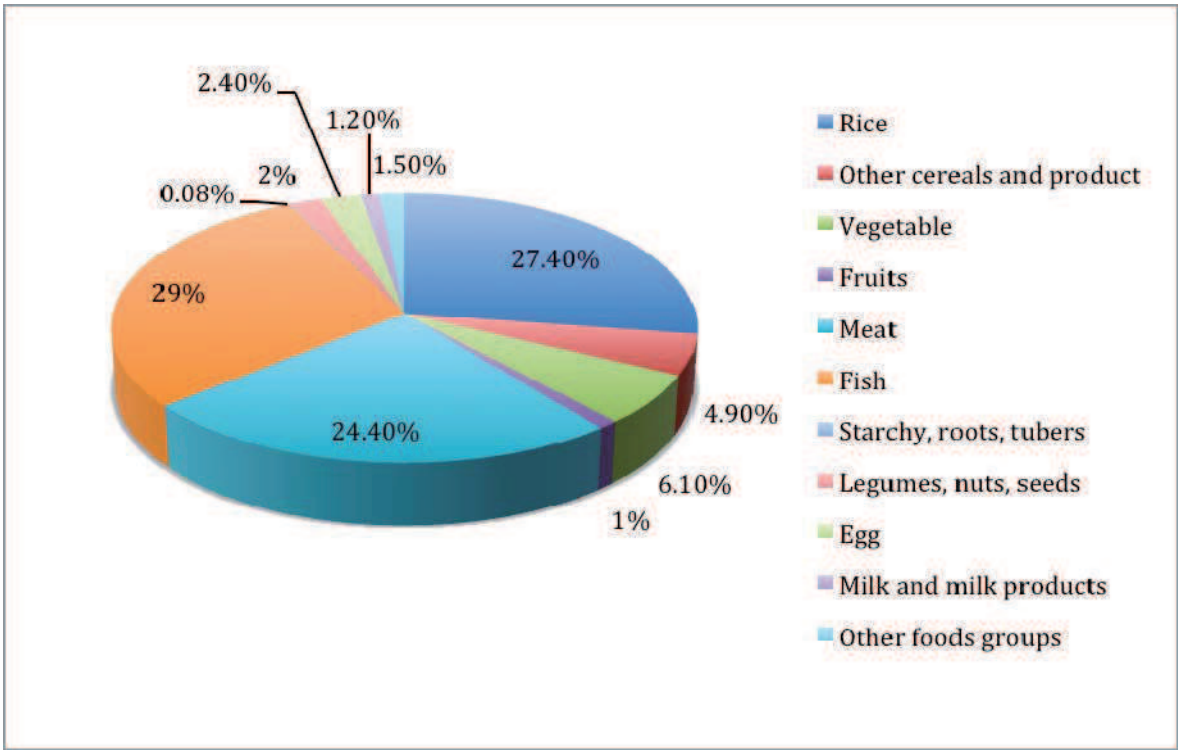


Figure 3.4. The major food groups contributed to the total protein intake.

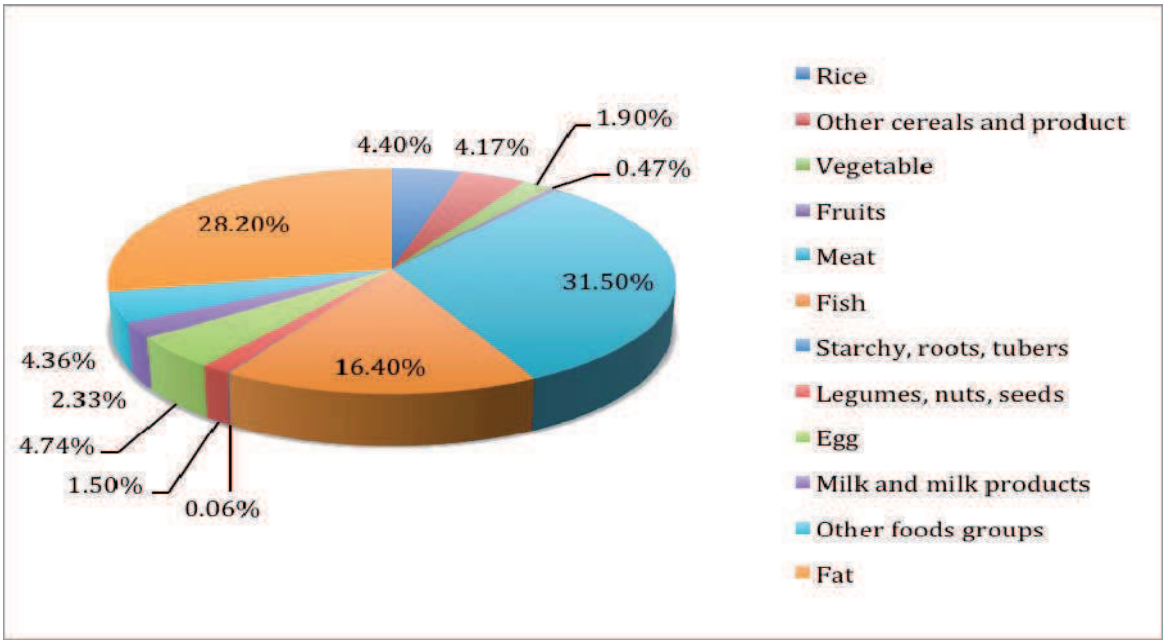


Figure 3.5. The major food groups contributed to the total fat intake.

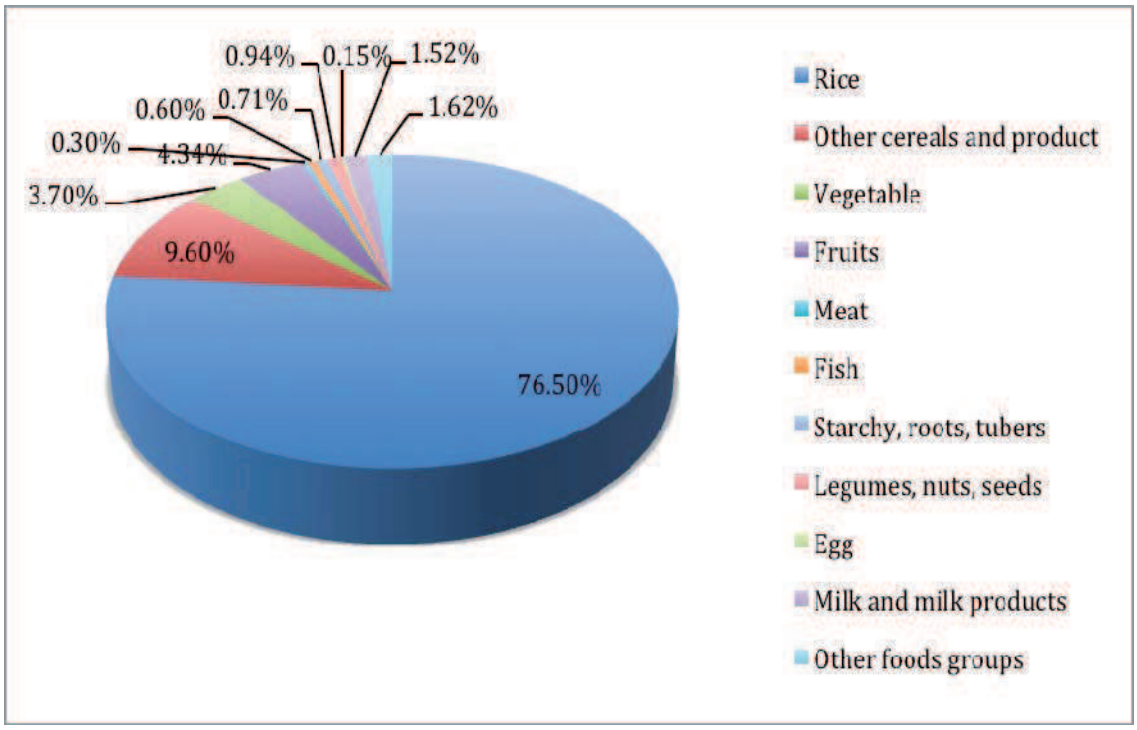


Figure 3.6. The major food groups contributed to the total intake of carbohydrate

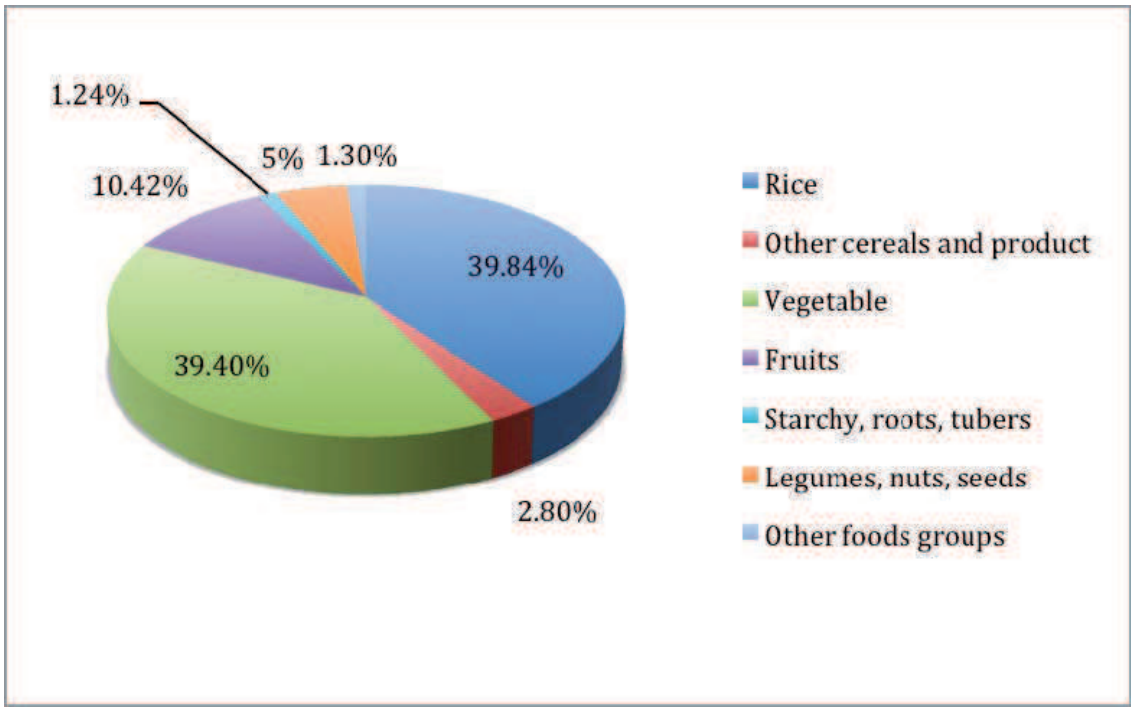


Figure 3.7. The major food groups contributed to the total intake of dietary fiber

Mean daily intake of fat for the whole population represents 17.5% of the total daily energy intake. Meat contributed with the highest percentage of fat intake (31.5%) follows by fats and oil (28.2%) and fish (16.4%). The mean carbohydrate intake was 331.9 g/person/day, in which contributed to

68.8% of the total energy intake. The top-ranked food sources of carbohydrate included rice (76.5%), other cereals (9.6%) and fruits (4.34%). The mean intake of dietary fiber was 12.4 g/person/day, which was closed to the lower end of recommended range (20-35 g/day). The top ranked food sources of total fiber intake they contributed were as follows: rice (39.84%), vegetable (39.4%) and fruits (10.42%). The major food sources of for minerals and vitamins were vegetable and fruits as presented in figure 3.8, 3.10 and 3.11. Similar to younger adults, the elderly obtained their iron from both plant sources and animal source: rice (26.34%) vegetables (22.2%, especially dark green vegetables), fish (12.75%) and meat (11.33%, especially pork/pork products) (**Fig.3.9**). Vegetable and fruits wee the most contributor to vitamins intake. Nearly 90% of vitamin A intake was from vegetable (**Fig. 3.10**); and 68% and 27% of vitamin C intake was from vegetable and fruits, respectively (**Fig. 3.11**).

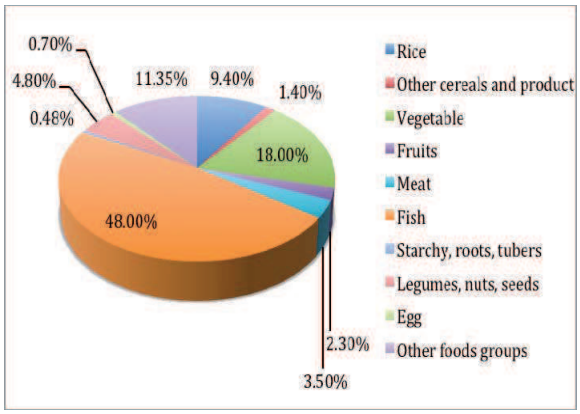


Figure 3.8. The major food groups contributed to the total intake of calcium.

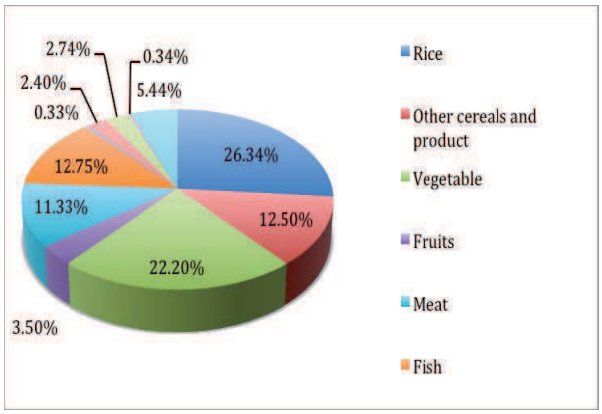


Figure 3.9. The major food groups contributed to the total intake of iron.

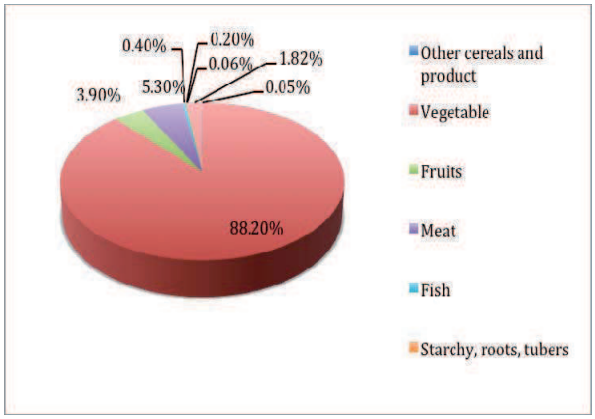


Figure 3.10. The major food groups contributed to the total intake of vitamin A

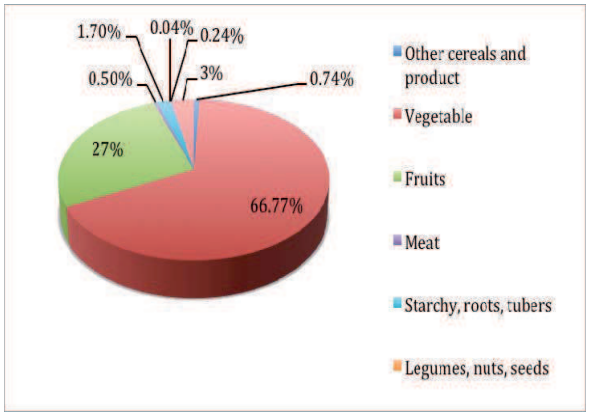


Figure 3.11. The major food groups contributed to the total intake of vitamin C.

4. Discussion

4.1. Dietary pattern in Cambodia

The present study is the first one to describe the eating patterns, seasonal differences, and eating variations in four different regions among the adult population in Cambodia. It showed that overweight and obesity represented 14 and 2.4% of the considered population respectively. At the same time, about 12% of the studied population was malnourished. In comparison, the prevalence of undernourishment or food deprivation as defined by the Millennium Development Goals and measured by Cambodia Socio-Economic Survey was estimated to be 23% in 2003-2004. The food deprivation among rural population was 24%, which was marginally higher than that of urban areas (22%). At sub national levels, the highest prevalence of food deprivation occurred in the lowest income population group, especially in women (NIS, 2007; FAO, 1999). However, the proportion of the population that is undernourished in Cambodia is known to have declined from 43 to 26% between 1990 and 2005, in parallel with economic improvement (Tuyen, 2009).

Higher food diversification was found in the Cambodian capital than in the other regions. Overall, food consumption of the Cambodian population was quite similar in the wet and dry seasons. The present results showed that rice is a staple consumption of the Cambodian population as in the other Asia countries such as Lao People's Democratic Republic, Viet Nam, Thailand, Myanmar and Bangladesh (Hop, 2003; Kennedy *et al*., 2002; Kosulwat, 2002; Norimah *et al*., 2008).

Vegetables represented in volume the second greatest foodstuff consumed in the investigated Cambodian population after rice. Vegetables constitute a major component of Cambodian cooking, present up to 70-80% in a dish. This can be attributed to the availability and accessibility of such products in all seasons and regions, as well as their high national production and relative low cost (Kanungsukkasem *et al*., 2009; Kosulwat, 2002; Ruel *et al*., 2005; Shetty, 2002).
Interestingly, participants in capital area (R1) were eating significantly more fruits than people from other regions. Fruit consumption seemed to increase in the capital with household income and education (Figuié, 2003; Satheannoppakao *et al*., 2009). On the contrary, on the southern coast (R4), consumption of fruits and vegetables was the lowest among all studied regions. In this region, vegetable and fruit production is low; therefore fruits and vegetables are mostly imported. As low fruit and vegetable consumption is among the top 10 risk factors contributing to mortality worldwide (WHO, 2003), public education and campaigns on adequate consumption of fruits and vegetables should be targeted more towards low socio-economic groups (Kanungsukkasem *et al.,* 2009; Satheannoppakao *et al*. 2009).

Meat and fish are both consumed daily by almost 100% of the studied Cambodian population. The production and the consumption of beef, pork and poultry have strongly increased in Cambodia since 1980 (Knips, 2004). The highest consumption of meat was observed in the capital (R1), with in the mean time the lowest fish consumption in this region. Traditionally, per capita consumption of non-ruminant meat in Cambodia as well as in Southeast Asia is higher than that of ruminant meat with the opposite being true in South Asia. This feature is generally accepted to be a consequence of the greater availability and lower relative prices of the dominant meat-type consumed (Rutherford, 1999). Processed meat such as ham, smoked bacon, meat sausage hamburger, hotdogs and varieties of canned meat are rarely found, other than in the fast food restaurants in the capital. The increase in processed meat and meat consumption in the capital of Cambodia is likely to be due to rapid development, as in other Asia Countries such as in Korea and Malaysia (Babji *et al*., 1995; Norimah *et al*., 2008; Son, 2003).

The highest consumption of fish and seafood observed in the area of the southern coast (R4) was likely to be related to aquaculture which is well developed in that region; fresh water fish from the Mekong River and Tonlé Sab lake contributed to the relatively high fish consumption in R2 and R3. Fish is the main source of proteins as well as of a number of vitamins and minerals (Roos *et al*., 2007a,b) for the Cambodian population. Obviously, fish is a major component of Cambodian diet, being an alternative to meat. Cambodia, with its productive inland fisheries, ranks among the top 25 countries in terms of aquaculture volume according to the latest FAO statistics (FAO, 2008). According to recent data source, the average per capita consumption of fish in all seven countries in Southeast Asia (including Cambodia) is higher than the world average of 16.1 kg a year (FAO, 2006; Hishamunda *et al.* 2009; World Bank, 2005). Our results show the same tendency, with an average fish consumption in Cambodia above the international recommendation which calls for cooked fish consumption of at least 150 g (5 ounces) or 2 servings per week as part of a healthy pattern of eating (EFSA, 2005; Canada, 2009; UK, 2004; US, 2005; FAO/WHO, 2003). It seems that the majority of the Cambodian population (97% of consumers in this study) consumes fish at least one time per day. Epidemiological studies have shown that fish consumption is associated with a reduced risk of coronary heart disease (Marckmann *et al*., 1999). It was estimated that in high-risk populations, an optimum fish consumption of 40-60 g/day would lead to approximately a 50% reduction in death from coronary heart disease.

The parallel development in the per capita consumption of meat and of fish (and seafood) seems to imply that there will be no shift in consumption patterns between the two products with rising

incomes. Additionally, indicators of modernization, particularly economic development, influence on the consumption of both meat and fish (York *et al*., 2004). On the other hand, the high price of meat in Cambodia and the availability of fish are partly responsible for the remaining high consumption of fish, particularly in the rural areas (FAO, 2000; Thang *et al*., 2004).

There was a significant difference in mean egg consumption between men and women in the studied Cambodian population (being respectively near 39 and 33 g/person/day for men and women), with a higher consumption in the Capital. Similar results were found in Singapore and Malaysia where men consume eggs more frequently than women (Norimah *et al*., 2008; Yeo, 1998). Compared to the other countries in the Mekong region, egg consumption in Cambodia was similar to that in Lao but less than in Thailand and Vietnam (Burgos *et al*., 2008, FAO, 2005).

Milk and dairy products do not belong to the traditional diet in Cambodia and are imported. However, in urban centers the demand for dairy products is increasing (Dong, 2005; Fabiosa, 2005; Steinfield *et al*., 2006). Our results show no difference in milk consumption among the studied population in terms of gender, season and region. Concentrated sweet milk remains the most widely used in Cambodia for coffee milk preparation. Imported pasteurized and sterilized milk is not very popular due to its high price. Yogurts are found in the supermarkets but they are not widely consumed by the studied population. We did not find cheese or other dairy products consumed by the studied population. Dairying is not a major industry in most developing Southeast and South Asian countries and therefore it is unable to offer much in terms of milk (Rutherford, 1999).

The estimated daily average consumptions of starchy roots and tubers, legumes, nuts and seeds, and sugar (32.2, 38.5 and 23 g/day, respectively) reported in this study were lower than the WHO estimates (119.7, 58.2 and 58.6 g/day, respectively). In contrast, the mean consumption value of fats and oils, condiments and spices were above WHO values (WHO, 2007). Sugar is usually added to beverages such as coffee and tea, and also to a meal. Cambodian cooking habits are salty and sweet test. There were no significant difference in sugar consumption between men and women on the wet and dry season.

Fats and oils are used for strewing, pan-frying and deep-frying, which is the main source of consumption for the Cambodian population. Fats and oils consumption did not vary between the wet and dry seasons. Fats, oils and sugar consumption were significantly different between R1-R2 ($p=0.04$), R2-R3 ($p=0.02$) and R2-R4 ($p=0.01$). Fats and oils consumption by the studied Cambodian population was 22.7g/person/day, which is in agreement with a previous study reporting that in

South and South East Asia consumption of fats and oils is less than 50 g/capita/day (Grigg, 2000). In Malaysia, fat intake among rural subjects was 42 g/day in males and 39 g/day in females, while in urban areas daily fat intake was 55 g in males and 64 g in females (Chee *et al*., 1997). The increase of fats and oils consumption was also noticed in the dietary pattern of Thailand (Kosulwat, 2002; Kwanbunjan *et al*., 2005). In Korea, fat consumption has increased with elevated consumption of animal foods and added fats and oils, with predominant increase in vegetable oil consumption (Son, 2003). However, subjects in this study consumed less fats and oils compared to the other Asia countries such as Malaysia and China (fat and oil consumption in China was 79 g/capita/day) (Chee *et al*., 1997, FAO/WHO, 2003).

In Cambodia, condiments and spices consumption was higher in women than in men. Also, significant differences in the consumption of condiments and spices were found between wet and dry seasons, as well as between R1-R3, R1-R4, R2-R3 and R2-R4. Soy sauce, fish sauce, monosodium glutamate and oyster oil are commonly found in Cambodian cooking.
The significant differences observed between men and women for fats and oils consumption as well as for condiments and spices are probably an artifact due to the better knowledge of recipes by women, leading to a better quantification of these foods used mainly as added ingredients.

In the 24-hour recall questionnaire, the percentage of consumers of alcoholic beverages was 5.6%. The average consumption of alcoholic beverages (255 and 548 g/day respectively for mean and high consumers) was higher in men than in women and during the dry season in comparison of the wet season. In terms of alcohol consumption (g of alcohol per day), we did not find any significant difference between seasons and sexes. The quantity of alcohol was 26 and 60 g/day respectively for the mean and the high consumption. In this study, the percentage of male consumers of alcoholic beverages was similar in all regions. However, obviously in rural areas the most consumed alcoholic beverage is rice wine (with a high degree of alcohol) and palm wine, while beer is more consumed in the capital and central areas (its high price may explain its lower consumption in rural areas).

4.2. Energy and nutrient intake in Cambodia

The reported energy intake of Cambodian adult population considered in this study (from different regions covering nearly the whole country) is on average 86.1 and 89.4% of RNI for men and women, respectively. The energy intake achievement in both sexes remains below the value of the recommended dietary allowances (RDAs) for Southeast Asia (Barba *et al*., 2008), and declines with age (Mirnalini *et al*., 2008). Distinct differences in energy intake can be seen amount seasons (p

≤0.01) and sexes, which is indicating that food habit may play a role (Chee *et al*., 1997). The mean distribution of macronutrients to the total energy intake amongst Cambodian population in both sexes was 13.7% for proteins, 17.5% for fat and 68.8% for carbohydrates, achieving the nutrient goals recommended by WHO and the Southeast Asia recommendation (Barba *et al*., 2008; Nishida *et al.,* 2004; FAO/WHO, 2003). We show that rice contributes to half (53%) of the total energy intake, being undoubtedly the stable food in Cambodia. Globally, the share of dietary energy supplied by cereals appears to have remained relatively stable over time, representing about 60% of dietary energy supply in the developing countries (FAO/WHO, 2003). Recently, however, subtle changes appear to be taking place. A closer analysis of the dietary energy intake shows a decrease in developing countries, where the share of energy derived from cereals has fallen from 60% to 54% in a period of only 10 years (WHO, 2003).

The distribution of energy from macronutrients in the diets of Cambodian from this study was found to be higher in carbohydrates (68.8%) than reported for Singaporeans (55%), Malaysians (59%), Hong Kong Chinese (53%), Japanese (59%), Korean (52.4%) and Thailand (61.2%). The energy intake from fat of Cambodian population (17.5%) was comparable with Japanese (16%) and lower than Singaporeans (30%), Malaysians (28%), Hong Kong Chinese (29%), Korean (34.9%) and Thailand (25%). In contrast, the energy intake from proteins (13.7%) of this studied population was low compared to that of these Asian Countries (Kwanbunjan *et al*., 2005; Mirnalini *et al*., 2008; Park *et al*., 2004). In comparison to the western countries, however, Cambodians consume proportionately more carbohydrates but less proteins and fat compared to British (carbohydrate 45%, protein 16%, fat 39%), Swedish, Australians (carbohydrate 45%, protein 17%, fat 32%), and New Zealanders (Becker, 1999; Mirnalini *et al*., 2008; Russell *et al*., 1999).

Percentage RNI achievement for calcium was 64% for adults in both sex and all age ranges, with significant differences noted among seasons. Hence, calcium intake in both men and women across regional groups did not meet the regional and international recommendations (Barba *et al*., 2008; FAO/WHO, 2003). However, it still remains above the calcium intake of Malaysian population (Mirnalini *et al*., 2008), as well as Thai population (Kwanbunjan *et al*., 2005; Pongchaiyakul *et al*., 2008). The major chronic diseases related to low calcium intake are kidney stones, colon cancer, breast cancer and hypertension (McCarron *et al*., 1999; Lipkin *et al*., 1999; Wu *et al*., 2002).

The average intake of iron among studied population was 12.5 mg for adult population, with a RNI achievement lower in women (44% of RNI) as compared to men (72.7% of RNI). Iron consumption in the studied population was found similar in all regions and among age ranges. In fact, iron

deficiency has been the most widespread nutritional disorder reported in Cambodia (Connoly *et al*., 2001). It is estimated that more that 70% of pregnant women and 74% of children under five years of age suffer from anaemia, mainly caused by iron deficiency (Connoly *et al*., 2001; Charles *et al*., 2009). In comparison, in Southeast Asia, iron deficiency and anaemia affect 50% or more of pregnant women (Ma *et al*., 2002; Seshadri, 2001).

The average mean of sodium daily intake was 2996 mg for adult Cambodian population in this study. Cambodian women consumed about 300 mg more than men, while the intake seems to be higher in wet season compared to dry season. Sodium intake has been consistently associated with blood pressure, which is a major risk factor for coronary heart disease and stroke (Gibbs *et al*., 2000; Law *et al*., 1991; WHO, 2003). The upper safe level of sodium intake is considered to be 2400 mg a day (Cutler *et al*., 1997; Graudal *et al*., 1998). As a result of the high salt intake around the world, in 2003, the WHO set a worldwide target of 5 g or less of salt (<2000 mg sodium) per day per person (WHO, 2003). In most Asian countries, the sodium intake is generally higher than this limitation (Brown *et al*., 2009; Kim *et al*., 2011). Culturally, in Cambodia as well as in the other ASEAN countries, an excess intake of sodium is observed due to the frequent use of several condiments such as soy sauce and fish sauce, as well as consumption of salted-fish which contains high concentrations of salt (Brown *et al*., 2009; Mirnalini *et al*., 2008; Takachi *et al*., 2010). However, this individual intake is still lower than generally observed in several developed countries such as France (3120 mg/day), Finland (3300 mg/day), Canada (3400 mg/day), USA (3436 mg/day) and UK (3460 mg/day) (Meneton *et al*., 2009; Reinivuo *et al*., 2006; Canada, 2004; US, 2008, UK., 2008). Indeed, in US and Europe, prepared and processed foods account for as much as 75% of daily sodium intake (Meneton *et al*., 2009; USDA, 1997).

The intake of vitamins among Cambodian population in this survey did not meet the daily reference intake of Southeast Asia nor the international recommendation (Barba *et al*., 2008; EFSA, 2006; FAO/WHO 2001), except for vitamin C, which reached the RNI. A detailed analysis of diets showed that one of the principle micronutrients sources in Cambodian diet was from fish. Freshwater fish is a dietary source of vitamin A and iron in Cambodia (Roos *et al*., 2007a,b). Low intakes of micronutrients, such as vitamin A, iron and zinc, are widespread, causing retarded growth and mental development in children, as well as high morbidity rates and increased risk of early death in other vulnerable population groups, such as women at the reproductive age (SCN, 2004) and were well noticed in Cambodia. Vitamin A deficiency and iron deficiency anaemia affect large numbers of the populations in Asia, often overlapping (Mason *et al*., 1999).

Thiamin is a water-soluble and heat-labile vitamin found in grain, roast pork, ham, nuts, catfish, and pasta. It acts as a coenzyme in the carbohydrate metabolism and possibly in nerve conduction (essential for the synthesis of acetylcholine) (FAO/WHO, 2001; Wu *et al*., 2005). There are two major manifestations of thiamine deficiency: cardiovascular disease (wet beriberi) and nervous system disease ("dry beriberi" and Wernicke-Korsakoff syndrome) (FAO/WHO, 2001; McCormick *et al*., 1994a). The mean thiamin daily intake of the studied Cambodian population was below the recommended intake.

Riboflavin (vitamin B2) is a water-soluble vitamin found in milk and other animal products (FAO/WHO 2001; Wu *et al*., 2005). It acts as a coenzyme in many reactions involved with carbohydrate metabolism. It is also needed to process amino acids and fats, activate vitamin B6 and folic acid, and help convert carbohydrates to energy (adenosine triphosphate ATP). Under some conditions, vitamin B2 can also act as an antioxidant (FAO/WHO, 2001). Riboflavin deficiency results in the condition of hypo- or ariboflavinosis, with sore throat; hyperaemia; oedema of the pharyngeal and oral mucous membranes; cheilosis; angular stomatitis; glossitis; seborrheic dermatitis; and normochromic, normocytic bone marrow (McCormick *et al*., 1997; McCormick *et al*., 1994b**)**. Those most vulnerable to deficiency of vitamin B2 include alcoholics, elderly persons, persons who suffer adverse reactions to dairy products (i.e. lactose intolerance), and women who use oral contraceptives. In this study, recommended daily intake (1.1 mg/day for women and 1.3 mg/day for men) was not fulfilled for riboflavin, which is in agreement with the fact that milk and dairy products are not widely use in Cambodia (FAO/WHO, 2001).

Niacin (nicotinic acid or nicotinamide) is essential in the form of the coenzymes nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP). It is found in dairy products, poultry, fish, lean meats, nuts, and eggs (FAO/WHO, 2001; Wu *et al*., 2005). NAD and NADP serve in many biological reactions such as intracellular respiration as well as in fatty acid and steroid synthesis (EFSA, 2006). Mean observed daily intake was around 90% of RNI.

Vitamin C is found in citrus fruits (such as oranges, limes, and grapefruits) and vegetables (including tomatoes, green pepper, potatoes) (EFSA, 2006; Naidu, 2003). It has a number of biochemical roles in the body (Iqbal *et al*., 2004). Particularly, it is a strong reducing agent and antioxidant, which is important in preventing the damaging effects of free radicals. Vitamin C is an enzyme co-factor for many biochemical reactions, especially those involving oxidations, such as the synthesis of hydroxyproline from proline for collagen biosynthesis, mono-oxygenases, dioxygenases and mixed function oxygenases. It is important in the synthesis and stabilisation of neurotransmitters and

carnitine, and increases the gastrointestinal absorption of non-haem iron by reducing ferric to ferrous iron (SCF, 1993). Hence, vitamin C facilitates the absorption of iron. In this study, it appears that the Cambodian population intake of vitamin C reaches the recommendations in both sexes (Barba *et al*., 2002).

In summary, our data support previous dietary surveys and studies, showing that a significant proportion of the population in many Asian countries has intakes below nationally recommended levels for several vitamins and minerals (Ma *et al*., 2002; Seshadri *et al*., 2001; Mason *et al*., 1999; Mirnalini *et al*., 2008) (Annexe III). This poses an increased risk of poor nutritional status for important nutrients, including iron, calcium, zinc, vitamin A, vitamins B1, B2 and niacin. The vitamins and minerals intake deficiency may be related to habitual diets (Slater *et al*., 2004). Rice and wheat are the staples for many populations of the world. Excessive refining and polishing of cereals removes considerable proportions of B vitamins contained in these cereals. Clinical manifestations of deficiency of some B vitamins such as beri-beri (cardiac and dry), peripheral neuropathies, pellagra, and oral and genital lesions (related to riboflavin deficiency)-were once major public health problems in parts of the world (FAO/WHO 2001). In Cambodia, polished and refined rice is a staple food that is served at least two serving sizes a day. So, eating unrefined rice should be promoted in Cambodia to avoid nutritional deficiencies, but this may be in conflict with flavouring and taste habits of the population. The food groups with a high contribution to vitamin intake were the following: vegetables for vitamin A; meat and cereals/roots for vitamin B1; dairy products, vegetables, cereals/roots and meat for vitamin B2; cereals/roots, seafood and meat for niacin and vegetables, and fruits for vitamin C (FAO/WHO, 2001). In Cambodia, fisheries sector is a major contributor to food and nutrition security, and fish has long been critical to all Cambodians. It is a major source of protein, minerals and vitamins and has become an integral part of the diet in Cambodia (Nam *et al*., 2011; Roos *et al*., 2007a,b; Victora *et al*., 2005).

According to the recommendations, ideal daily intake of calories for the general population from protein, lipid and carbohydrates would be 10-14%, 20-30%, and 58-68%, respectively. The results of our survey showed that the intake of carbohydrates (68.8%) in the adult population was reach to the higher to than this recommended level, whereas the caloric intake from protein (13.7%) was at the recommended level, and the intake of calories from lipid (17.5%) was in the middle range of the recommended level.

The daily intake levels of the energy and three macronutrients are shown in Table 3.10. Rice was the major contributor of the total energy intake (53%) as well as the macronutrients intake (76.5%,

27.4% and 39.84% for carbohydrate, protein and fiber, respectively). Vegetable and fruits were the most contributor of the total intake of vitamins and minerals. The food contribution patterns for macronutrients differed between the elderly and younger adults in the several difference food groups' consumption. The elderly (55-65 years old) consumed less rice, meat and eggs and eggs products but consumed more milk and milk products and fish than younger adults (25-34 years old). These patterns may contribute to lower lipid and protein intakes in the elderly and highest intake in calcium due to the high intake of fish (fish contributed 48% of the total calcium intake). In general, vegetable and fruits were the contributor to vitamins intake in these studied population.

4.3. Strengths and limitations of the present study

The advantage of the 24-hour recall is to provide accurate and quantified listings of all foods and beverages consumed during the 24-hour period before the interview. In addition, repeating the test twice within three days allowed us to account for the intra-individual variability of the consumption. Besides, specific FFQ were developed mainly to assess the long-term consumption, cooking methods and doneness preferences for meat, poultry, fish, sausage and offal products which are relatively frequently (meat, poultry and fish) and infrequently (sausage and offal products) consumed in Cambodia.

Nevertheless, several limitations of our study must be addressed. One is the evaluation of the seasonal variation that was carried out by repeating the questionnaires twice, once during the wet season and another time during the dry season. This approach, chosen due to obvious problems of resources, could have been insufficient to capture all the seasonal variations. The seasons during which the survey took place (March/September) could lead to an overestimation of consumption of certain food items such alcoholic beverages, fruits and vegetables. Also, the number of male participants was limited because interviews were conducted at home and women are more frequently at home than men. Consequently, the gender difference could be affected by this limitation. For the same reason, follow up of male subjects for the second cycle of the survey was very difficult.

5. Conclusion

In summary, as in other ASEAN countries, rice remains the staple food for our studied Cambodian population. Dietary patterns in the capital seemed to be more diversified than in the rural areas, and, in particular included more meat products as well as more fruits and vegetables. Similarly the consumption of fish was notably high compared with other countries around the world, and a shift between meat and fish was only marginally observed in the Capital city. Overall, dietary habits of the

Cambodian population have not dramatically changed towards a more western style diet. The consumption of rice declined with income growth, while consumption of animal meat, poultry, eggs, fats, soft drinks and beer slightly increased. Our data in terms of energy and nutrient intake are indicative of a full picture of the current situation in Cambodia, and provide unique insight on the energy and selected nutrient intakes of Cambodian population. Obviously, the daily energy intake does not fulfill the recommended daily intake for both men and women, although intake of macronutrients met the recommendations for the healthy diet. The intake of micronutrients studied was below the RNI for iron, calcium, vitamin A, vitamin B1 and B2. The worse deficiency was observed for iron, particularly in women. Therefore, the inadequacies of dietary intake in rural areas, leading to nutrient deficiency, is still of concern in Cambodia, especially for women. Effective policies, programs, and initiatives to promote adequate diversification of food consumption in rural areas are needed. Improving the economy of the poor is important as low nutrient intakes are closely linked to poverty in the rural areas. Regular nutrition surveys should be carried out in order to provide valuable information on trends in food and nutrient intake.

With regards to CRC, our data do confirm lifestyle as to be potentially protective against CRC development; adaptation of western-style diet occurred only in a minority of the study cohort, mainly in those living in the capital city. An abundance cereal and plant food contributed to provide a higher of carbohydrate and dietary fiber intake of the Cambodia population and low consumption of total fat. Fish consumption in varying amount that is the highest consumption amount the other countries, which provided several essential vitamins and mineral. The richest of plant food and cereal and fish in Cambodian dietary pattern may contain many protective factors of the chronic disease such as cancer. Yet, future studies are needed to explore the associations between future trends in dietary intake and chronic diseases among Cambodian population.

Chapter 4. Dietary exposure assessment of Cambodian population to some colorectal carcinogens

1. Introduction

As discussed previously, humans are exposed through their diet to complex mixtures of substances that may be involved in causing, modulating, or preventing cancer. In **Chapter 1** we have detailed reported results, from either epidemiological or rodent studies, giving evidence of a strong link between diet and CRC. It is now well-established that the risk for CRC is strongly related to the consumption frequency of meat as well as to the cooking preference (a higher risk being related to the consumption of grilled, barbecued or fried well-done and very well-done meats). Since 1990, near twenty studies have been published on the subject; most studies (but not all) confirm that the intake of grilled, barbecued, fried and/or well-done red meat is more related to CRC risk than the intake of total red meat (Cotterchio *et al*., 2008; Girard *et al.*, 2008; Nowell *et al.*, 2002; Rohrmann *et al*., 2002; Shin *et al.*, 2007a; Sinha *et al*., 1998a,b). For instance, well-done cooked meat and fried meat intakes have been positively associated with CRC in some case–control studies (Butler *et al*., 2003; Gerhardsson *et al*., 1991; Lang *et al*., 1994). However, cohort studies have not substantiated these findings (Kabat *et al.*, 2007; Nothlings *et al.*, 2009; Pietinen *et al.*, 1999).

As mentioned earlier, among potential dietary carcinogens, there are two groups of compounds of high concern, namely HAs and PAHs; such compounds are not naturally present in foods and may be formed during cooking or preservation or can derive from environmental contaminations (PAHs). In particular, cooking food under high temperature results in the formation of HAs and PAHs, which adhere to the surface of the food (Navarro *et al.,* 2004; Sinha *et al*., 1999). These results are consistent with the hypothesis that carcinogenic compounds formed by high-temperature cooking techniques (such as HAs and PAHs) may contribute to the risk of developing CRC (Gunter *et al.,* 2005; Navarro *et al*., 2004; Sinha *et al*., 1999; Sinha *et al*., 2001; Sinha *et al*., 2005c). As PAHs are ubiquitous in the environment, they are present in almost all food. PAHs are usually found as a mixture containing two or more of these compounds. These contaminants generate considerable interest because some of them are highly carcinogenic in laboratory animals and have been implicated in breast, lung, and colon cancers in humans (Pufulete *et al*., 2004; Ramesh *et al*., 2004; Okona-Mensah *et al*., 2005) and BaP has been lassified as a possible human carcinogens (IARC, 2004).

To investigate the CRC risk due to HAs and PAHs in humans, an accurate estimation of exposure is needed (Sinha *et al.*, 2002). Besides, in spite of abundant research studies on the relationship between cancer risk and HAs present in typical Western cuisine (Augustsson *et al.*, 1997), with an emphasis on meats in general and red meats in particular, less is known about Asian cuisine with regards to its HAs content (Koh *et al.*, 2005). This is quite a challenge since exposure to HAs varies greatly between populations, depending on dietary preferences and cooking methods. As the main route of human exposure to such compounds is through consumption of meat and fish, we needed to obtain information on usual levels of meat and fish consumption by methodologies similar to those used to assess other components of diet (Sinha *et al.*, 1997). In addition, contamination levels of meat and fish are also required. Indeed, even though most studies have concentrated on investigating the influence of different cooking conditions on HAs formation and have proposed advices to limit their formation in cooked foods, cooking practices that favour their formation are still used in the cuisines of certain cultures, and may, conceivably, impact those populations by presenting various exposure to such carcinogenic contaminants. It has been reported that PAHs are present in various food products as they are produced during cooking at high temperature or may be due to environmental contamination of the food chain (White *et al.*, 2008; EFSA, 2008). Dietary exposure assessment of PAHs should focus on all food categories.

Cambodia is one of the developing countries in which the characteristic dietary pattern is not yet adapted to the western countries (being low in fat and red meat consumption, with simultaneous high carbohydrate consumption). Also, as already pointed out, Cambodia has an unusual age-distribution pattern of CRC, with the proportion of diagnostic cases under the age of 40 being ten times higher than observed in western countries (Hav *et al.,* 2011). Many investigations have suggested a possible role of genetic and environmental factors (such as pesticides) to explain this observation (Pratt *et al.*, 1977; Soliman *et al.*, 1997). Yet, to date there has been no study conducted in Cambodia to investigate the role of diet and dietary carcinogens on CRC in any age group. So, it is of primary importance to gain knowledge relative to dietary exposure assessment of carcinogens, especially those encountered in meat and fish products. In such a context, this work, focusing on dietary exposure assessment of HAs and PAHs constitutes the first step towards a better understanding of their possible adverse effect on population health (especially CRC). Afterward, the estimated individual HAs and PAHs concentrations in the diet have to be combined with available consumption data (Kroes *et al.*, 2002).

The purpose of this chapter is to present an intake assessment of three major (2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine: PhIP, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline: MeIQx and

2-amino-3,4,8-dimethylimidazo[4,5-*f*]quinoxaline: DiMeIQx or 4-8-DiMeIQx) and one PAH (benzo[a]pyrene: BaP), given to their toxicity and frequent analysis in food through respectively meat/fish and several specific food items consumption by the Cambodian population. Our investigations were aimed at carrying out a deterministic dietary exposure assessment in order to compare dietary exposure to these contaminants with toxicological reference doses to determine whether BaP and HAs exposure is or is not a matter of concern for the Cambodian population. By looking at the dietary exposure assessment, we aimed to identify typical dietary patterns that could partly explain the CRC incidence rates observed among the Cambodian population, especially among the young ones (less than 40 years).

2. Material and methods

2.1. Consumption data

Consumption data were obtained from FFQ and 24-hour recall surveys conducted in this work.

2.1.1. Food consumption data for HAs

A cross sectional survey was conducted during both wet and dry seasons in four different regions covering nearly the whole country as detailed in **Chapter 3**. A total of 941 respondents (age 25 and above) was selected randomly for the survey. A food frequency questionnaire (FFQ) was used to estimate the intake of meat and fish, taking into account different cooking methods and doneness. This FFQ included the information of four cooking methods (grilled, pan-fried, deep-fried and baked), four doneness levels (rare, medium, well-done and very well-done), five consumption frequencies (one time per day (1T/D), one time per week (1T/W), two times per week (2T/W), three times per week (3T/W) and one time per month (1T/M) as well as the portion size of meat and fish items. In the Cambodian diet, meat, poultry and fish dishes are cooked using several marinated methods that provide a suitable flavour; therefore the information on marinated meat, poultry and fish was also collected during the survey (see **Chapter 3** for detail of the survey). Finally, a total of 100 meat and fish items (marinated or not) was added to the FFQ. Before the main survey, a pilot survey with 40 subjects was performed in the Cambodian capital to test the questionnaire. The subjects were visited at home.

Data from the FFQ were used to determine meat / fish consumption frequency, portion size, cooking technique and doneness level. These data were used in conjunction with an established database of

HAs concentrations found for a variety of meats / fishes (cooked by different methods to a range of doneness levels) to estimate exposure to these food-related mutagens.

2.1.2. Food consumption data for BaP

Consumption data for the general population of Cambodia were obtained from 24-hour recall interviews conducted among a random sample of 941 participants of the four main principal regions in Cambodia (see **Chapter 3**); the population was divided into four age groups as already presented. For each food item consumed, the name of the dish, type of cooking method used and portion size (small, medium or large) of each dish were elicited. Most Cambodian dishes are mixed dishes with reasonably standard recipes (Long *et al*., 2010). Finally, the consumptions of a total of 250 food items from this 24-hour recall questionnaire (in gram/person/day) have been recorded into the Excel database from this 24-hour recall questionnaire for further dietary exposure assessment of BaP.

2.2. Contaminant database

Mean levels of food contamination for HAs and BaP were estimated based on selected data reported in the literature.

2.2.1. HAs data

The first quantitative data on HAs found in various meat and fish products based on chromatographic techniques was published in the late 1980s. The complex food matrix, the low amounts of HAs present and the need for several isolation steps made an accurate quantification difficult, but effective new methods for extraction, purification and detection of HAs have been recently developed (Pais *et al.*, 2000; Ristic *et al*., 2004). Amount these analytical techniques, high-performance liquid chromatography coupled to ultraviolet detection or preferably mass spectrometry is recommended as the most suitable technique for the analysis of a large number of HAs in food samples (Ericson *et al.*, 2007; Santos *et al.*, 2004).

There is an extensive literature on the presence of HAs in meat and fish cooked under high temperature, but many of the reported data are from food samples which were cooked to maximize HAs production (Adamson, 1990; Layton *et al.*, 1995) and are unlikely to be representative of the way meat and fish are usually cooked by the general population. Fortunately, some studies report HAs levels in food cooked by methods more representative of cooking practices (Sinha *et al.*, 1999). Iwasaki *et al.* measured HAs concentrations in fresh and marinated beef, chicken, pork, hamburger, sausage and fish cooked using different methods (pan-fried, grilled and churrasco typically used in Brazil) and to four levels of doneness (rare, medium, well-done and very well-done) by using liquid

chromatography-mass spectrometry analysis (Iwasaki *et al.*, 2010). Other authors have analyzed in Singapore, fresh and marinated meat 'chicken, pork, duck and beef) and fish samples (fish and seafoods,) cooked differently (Salmon *et al.*, 2006; Wong *et al.*, 2005). Another study has reported in Malaysia contamination levels in about 42 food items (meat, poultry and fish) cooked by different methods (Jahurul *et al.*, 2010).

Based on the available published data (about 15 publications), we have created an Excel database of HAs concentrations (PhIP, MeIQx and DiMeIQx) in 10 types of meat and fish (fresh and marinated) taking into account the cooking method and the degree of doneness that were included in the consumer behavior/preference survey. Overall, approximately 200 individual meat/fish items were considered in that database (Gross *et al.*, 1993; Iwasaki *et al.*, 2010; Jahurul *et al.*, 2010; Jo *et al.*, 2008; Keating *et al.*, 1999; 2001; Knize *et al.*, 1994, 1998; Liao *et al.*, 2010; Oz *et al.*, 2007, 2011; Salmon *et al.*, 1997, 2006; Sinha *et al.*, 1998a; Skog *et al.*, 1995a, 1998, 2002; Solyakov *et al.*, 2002; Wong *et al.*, 2005). When the data were gathered from reviews, the following information was extracted from each publication: (i) food information - food name, cooking method (including temperature and cooking time if available) and degree of doneness; (ii) measurement information - level of contamination, type of value (mean, median, range, maximum or other) and analytical method.

2.2.2. BaP data

In non-smokers, diet is the primary source of human exposure to PAHs, contributing to more than 90% of total exposure (McGrath *et al.*, 2007; SCF, 2002; WHO, 1998). As PAHs are ubiquitous in the environment, it is not surprising that they are present in almost all food. The presence of PAHs in foodstuffs depends on the environmental concentrations of these pollutants, as well as the physiological and ecological characteristics of the product (Ramesh *et al.*, 2004; Wenzl *et al.*, 2006). However, levels of PAHs are rather low in most raw food. On the opposite, food cooked under high temperature (especially meat and meat products, and processed meat) may contain higher concentrations of PAHs (Lorenzo *et al.*, 2010; Phillips *et al.*, 1999, SCF, 2002).

In order to assess the exposure to PAHs, BaP has been chosen as an indicator of PAH content; this choice was partly driven by the lack of reported contamination levels in foodstuffs for other PAHs. Due to inconsistent presence and strength of associations between dietary BaP exposure and cancers, there is a need to get reliable exposure data.

Based on the available published data (we collected about 10 recent publications), we created a database for BaP levels in various foodstuffs; in these studies, high-performance liquid chromatography was the method of choice for BaP analysis (Kazerouni *et al.*, 2001; Dennis *et al.*,

1983, 1991; EFSA, 2008; Falco *et al.*, 2003; Kayali-Sayadi *et al.*, 1998, 1999; Lin *et al.*, 2005; Lodovici *et al.*, 1995; Marti-Cid *et al.*, 2008; Martorell *et al.*, 2010; Miller., 2009; Orecchio *et al.*, 2009; SCF, 2002; Yoon *et al.*, 2007). We did not use data from studies that were based upon determination in food samples exclusively in highly contaminated areas, as in most cases, the purpose of such research was to monitor environmental pollution. The available measurement information included analytical method and type of values (e.g. minimum, mean, median, 95th percentile or maximum).

In the case of rice, BaP contamination data available in the literature were only found for raw rice. However, in our food consumption study, rice was recorded as cooked rice. So, to extrapolate the BaP level in cooked rice, we have made the following assumptions: (i) the water used to cook rice was not contaminated by BaP, (ii) the volume of cooked rice was increased three times when compared with the uncooked rice. Consequently, the BaP level in cooked rice (expressed in ng/g of food item) has been estimated as being one third of the level in raw rice.

2.3. Approaches for dietary exposure to HAs and BaP

Dietary exposure assessment is one of the processes in risk assessment. Emphasis on public health and consumer protection, in combination with globalisation of the food market, has created a strong demand for exposure assessment of chemicals. Dietary exposure assessment is the qualitative and/or quantitative evaluation of the likely intake of chemicals (including nutrients) *via* food, beverage, drinking water, and food supplements. A wide range of methodologies exists for estimating exposure to food chemicals, and the method chosen for a particular exposure assessment is influenced by the nature of the chemicals, the purpose of assessment and the available resources.

2.3.1. General formula

Assessment of exposure to food chemicals usually require some extent of modelling because, with the exception of duplicate diet surveys, the food consumption and chemical concentration data are not related to the same individuals within population. While food consumption data are usually obtained from national food consumption surveys, the chemical concentration data may be obtained from various sources (such as manufacturers, public analytical laboratories, field trials or literature). So, the assessor must make a decision about how to combine the food consumption data with the chemical concentration data to create a representation of the real-life situation.

In its broadest sense, the general model to represent dietary exposure assessment can be considered as the following formula:

$$E_i = \frac{1}{n_i\, bw_i} \sum Q_{i,t,k} * C_{i,t,k}$$

where: i is an index for individual

E_i is the chemical intake assessed for individual i (ng/kg b.w/day)

t is an index for time window to assess food consumption and contamination: day or single occasional of consumption (day)

$Q_{i,t,k}$ is the consumption of food group k on occasion t by individual i (kg)

$C_{i,t,k}$ is the contamination level of food group k encountered on occasion t by individual i (ng/kg)

k is is an index for food group: k=(1,…..14),

n_i is the number of days of food records available for individual i

$b.w_i$ is the body weight for individual i (kg)

The focus of dietary risk assessment has generally been on the risks arising from chronic (long-term) dietary exposure. Chronic exposure is a repeated exposure to low, or very low, doses for a long time. The chronic HAs and BaP exposure is the amount of HAs and BaP daily ingested from the daily consumption of all food items. For HAs and BaP, daily intakes were calculated individually for each food item.

Then, two approaches may be used to assess exposure: the deterministic approach (using point values) or the probabilistic (also called stochastic) approach (using distributions) (FAO/WHO, 2005, 2009). In that work data were not sufficient to perform a robust probabilistic assessment and therefore we combined the distribution of food consumption collected within the Cambodian population with the average levels of contamination from the public literature. This deterministic approach is often called distributional.

The distributional approach multiplies a series of fixed value for food consumption (usually the mean population value) by a fixed value for chemical concentration in that food (usually the mean concentration or maximum permitted level), and then sums the intake from all foods (Lambe, 2002). Such approach is commonly used as a first step in assessing exposure because it is relatively simple and inexpensive to carry out (Counil *et al.*, 2005; Kroes *et al.*, 2002; Lambe, 2002; Tressou *et al.*, 2004). Inherent in the point-estimate approach are the assumptions that (i) The assessment is only valid for individuals consuming the specified food(s) at the considered level, (ii) the food chemical is

always present in the food(s), (iii) the chemical is always present at an average or high level. This approach, therefore, does not provide a full distribution of possible exposures that may occur within a population, and does not allow a sensitivity analysis to identify the main factors influencing the results of the assessment.

In the present work, for each food item, the mean value of contamination data reported in the recent publications has been chosen. For example, in the case of BaP, we took the mean value of 0.1 ng/g in rice for the calculation.

Also, to estimate the mean concentration levels from data sets with variable proportion of value below the limit of detection (LOD) of the analytical method, we chose two scenarios (following the recommendations from the WHO GEMS/Food-EURO workshop) (WHO, 1995):

- Scenario 1: the non-detected values are replaced by zero (lower bound)
- Scenario 2: the non-detected values are replaced by LOD (upper bound).

2.3.2. Data analysis

Dietary exposure assessment of HAs and BaP of each subject has been calculated by multiplying individual food consumption data, which were obtained from FFQ for HAs and 24 hours recall for BaP, by the concentration data (mean value) of HAs and BaP. The intakes from all food sources were finally summed. The dietary intake of HAs and BaP, firstly, express in ng/person/day was divided by the body weight of the participants to be expressed as ng/kg b.w/day.

Finally, the exposure assessment of the contaminants has been analysis according to their food source in order to identify the potential source of contribution. The contribution of the each food group has been calculated express by the percentage of the distribution.

Individual consumption of food was calculated in g/person/day, and food contaminants intake level was calculated both in ng/person/day and ng/kg b.w/day. The descriptive statistic (mean, standard deviation and 95th percentile) of the HAs and BaP dietary exposure has been calculated. The mean, standard deviation and 95th percentile of the exposure with HAs and BaP were derived for the total participants, for gender, season, region and age-range. The difference significant between groups has been identified by look at to the p-value. All statistical analyses were performed using SPSS software, version 19. An independent T-test was performed for the normal distribution data and Kruskal-Wallis test was performed for the non-parametric data.

3. Results

3.1. Exposure assessment to BaP

3.1.1. Deterministic approach

In **Table 4.1** we present the main results (means, standard deviations and 95th percentiles) of the measurements of exposure to BaP issued from the deterministic approach. The distribution of the deterministic exposure assessment of the 941 Cambodian populations is presented in figure 4.1 and the other distribution of BaP exposure assessment for sex; season and regions were presented in annex IV.

Table 4.1. Dietary exposure to BaP in a Cambodian population (n=941).

Dietary exposure to BaP	**Mean ±SD**	**95th p**	**Mean ±SD**	**95th p**	**p-value**
	ng/person/day		**ng/kg bw/day**		
General population	55.6± 16.3	84.7	1.01±0.3	1.6	
Men	57.1±0.93	87.6	0.96±0.3	1.5	0.001
Women	54.7±0.64	83.8	1.04±0.34	1.7	
Season					
Dry season	53.8±12.5	84.5	0.96±0.3	1.6	0.001
Wet season	57.6±15.4	85.6	1.07±0.3	1.65	
Region					
R1	60.3±15.8	90	1.1±0.3	1.7	<0.05*
R2	55.3±11.8	84.8	1±0.35	1.7	
R3	55±15.7	84.5	1±0.3	1.6	
R4	50.1±14.8	75.4	0.9±0.26	1.14	
Age range					
25-34	58.5±16.3	88	1.1±0.34	1.7	<0.05**
35-44	54.6±15.2	83.3	0.97±0.3	1.4	
45-54	52.7±15.4	77.4	0.9±0.3	1.4	
55-65	53.5±17.8	83.8	0.97±0.36	1.7	

**: Independent T-Test performed between R1 with R2, R3 and R4; **: Test performed between age range 25-34 with 35-44, 45-54 and 55-65; p-value < 0.05: difference significantly between groups*
b.w: body weight (56.07 kg mean body weight for general population, 54 kg for women and 60 kg for men).

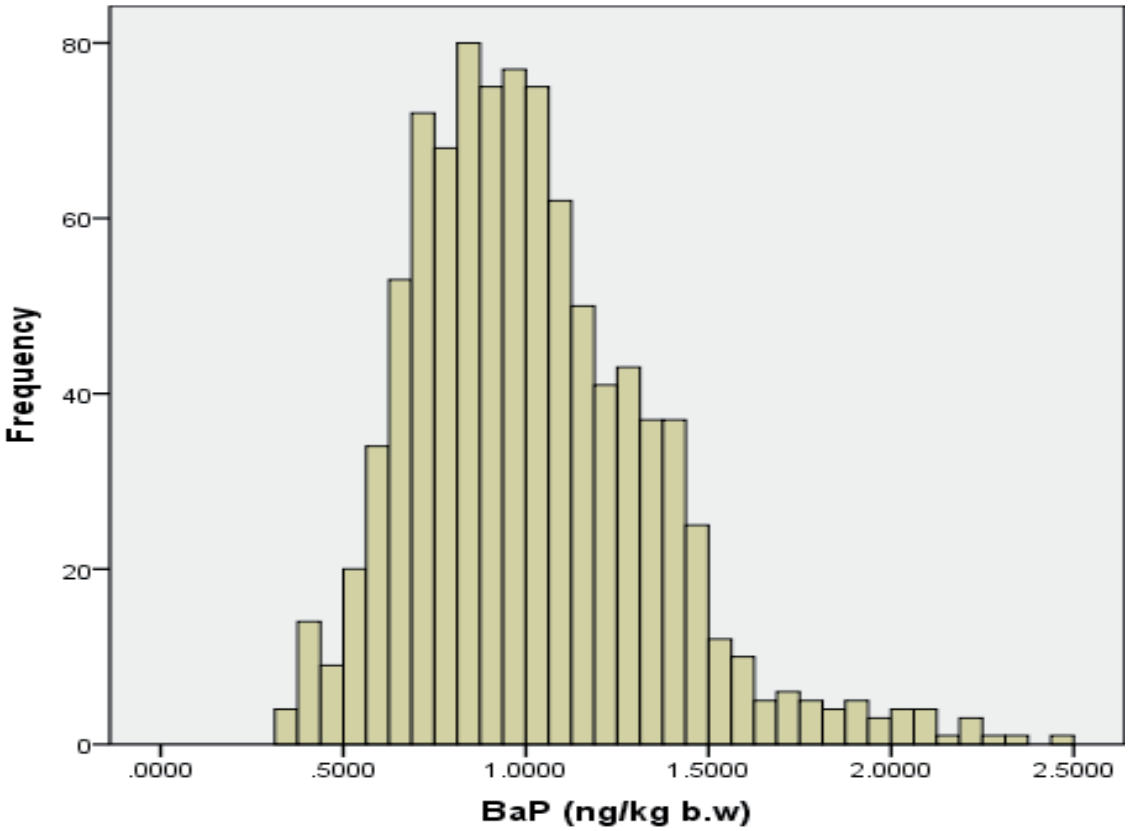


Figure 4.1. Estimated total dietary exposure to BaP (ng/kg b.w/day) for the Cambodian population using the deterministic approach.

Depending on the consumption data from a food consumption survey in a Cambodian population (n=941 individuals) and contamination values in the database we built based on literature data, an individual approach was applied for the BaP intake estimate. The mean values of exposure levels of adults are 55.6 ng/person/day (assuming a mean body weight of 56.07 kg) for the general population considered; the mean total dietary intake is difference significantly between the gender considered (males or females) ($p < 0.05$). On a kilogram of body weight basis, the assessed mean intake of BaP was 0.96 ng/kg b.w/day and 1.04 ng/kg b.w/day for men and women, respectively. The high exposure (95^{th} percentile) was 1.6 ng/kg b.w/day for the general population and 1.5 and 1.7 ng/kg b.w/day for men and women, respectively.

Interestingly, the values of BaP intake increased for the first age range compared to the other age ranges. Hence, the highest intake of BaP was found in the first age range (1.1 ng/kg b.w/day) whereas intake of BaP was found identical, in the other three age ranges (0.9-0.97 ng/kg b.w/day) ; the observed difference between the first age range and the other age ranges was statistically significant ($p < 0.05$).

Looking at differences due to location, it appears that the Capital population is slightly more exposed to BaP (1.1 ng/kg b.w/day) than the rest of the population (0.9-1 ng/kg b.w/day), the difference being statistically significant (p=0.001). Also, the season was found to have a significant influence (p=0.001) on BaP dietary intake, the Cambodian population exposure to BaP being higher in the wet season (mean intake 1.07 ng/kg b.w/day) than during the dry season, (mean intake 0.96 ng/kg b.w/day).

3.1.2. Food categories contributing the most to the BaP intake

The differential contributions to the BaP exposure of several food categories are presented in **Figure 4.2**. According to these food categories, the greatest contribution was from "vegetables and products" (27.35%), followed by "cereal and products" (18.5%), "fat and oil" (17.9%) "meat and products" (17.4%) and "fish, shellfish and products" (9.1%).

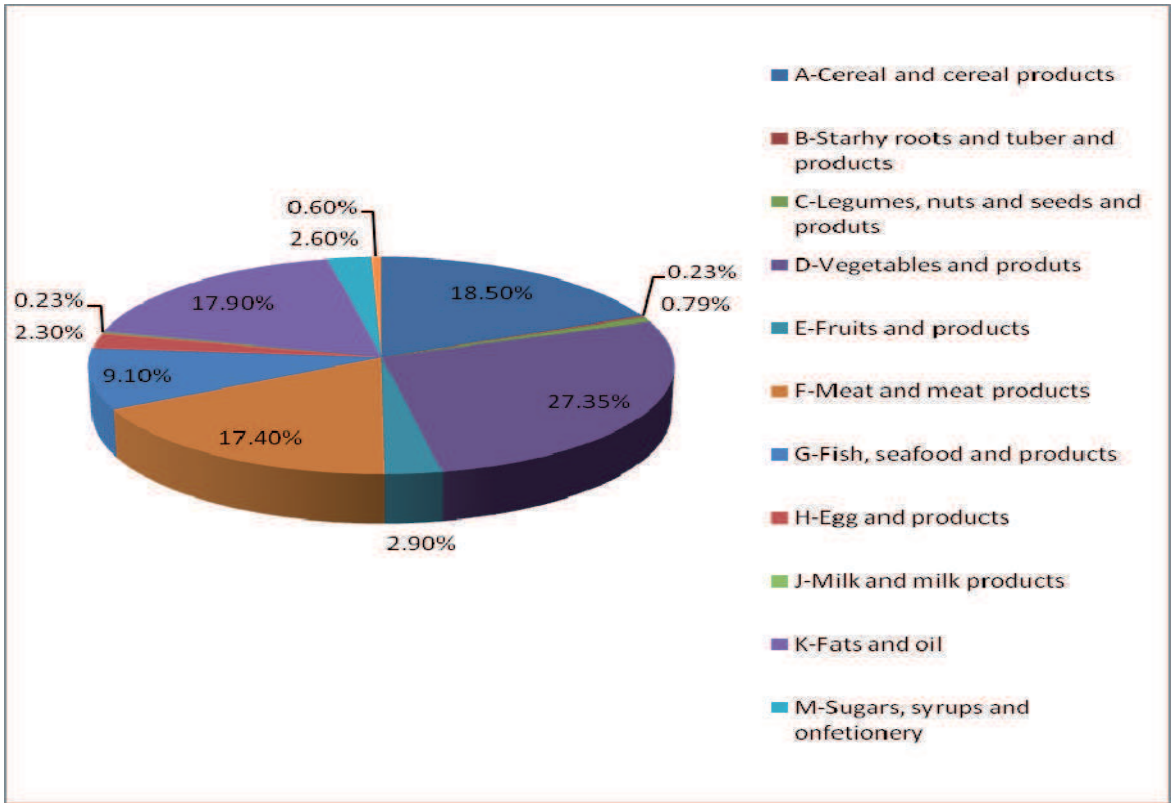


Figure 4.2. Contribution (%) of the different food categories to BaP total dietary intake.

In the cereal group, rice was the highest contributor (84.3%) (see **Figure 4.3**). Vegetable was major food group in contribution of BaP in the total dietary intake, in which, green vegetable represented about 34.8% follow by fruit vegetables (20.3%) (**see Figure 4.4**)

In the meat group, pork was the most contributor (62%) followed by beef (21.7%) (**see figure 4.4**). For the fish/shellfish group, fish (43.7%), grilled fish (15.6%), frying fish (11.9%), dried salted fish, seafood (8.4%) and fermented fish (7.9%) covered more than 50% of the total fish/shellfish contribution (**see Figure 4.5 and 4.6**).

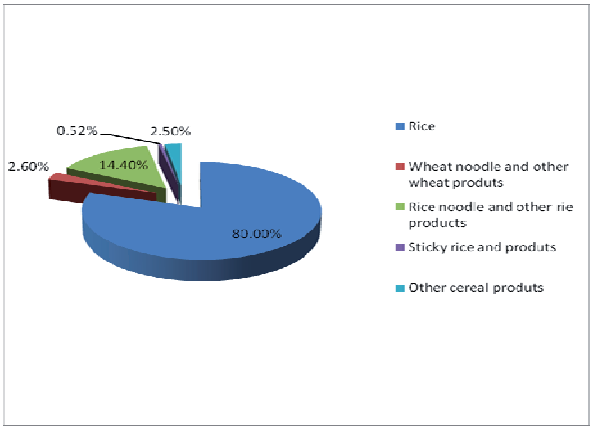


Figure 4.3. Contribution (%) of Cereal and products to BaP total dietary intake.

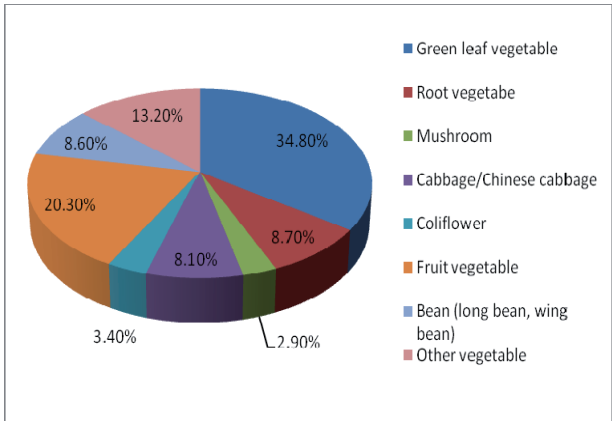


Figure 4.4. Contribution (%) of vegetable and products to BaP total dietary intake.

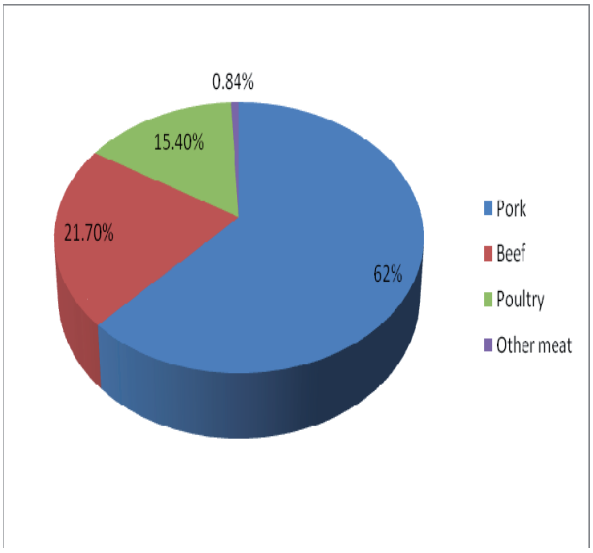


Figure 4.5. Contribution (%) of meat group to total BaP dietary intake

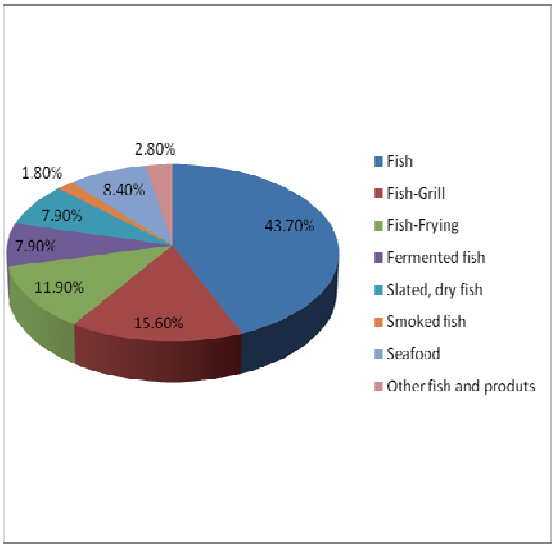


Figure 4.6. Contribution of fish group to total BaP dietary intake.

There was a similar contribution of the "cereal and products" category for the four age groups in the exposure to BaP. Meat contributed more to the BaP exposure in the younger age groups (25-34 and 35-44) than in the older age groups (45-65). In contrast, vegetables and fish/shellfish contributed more to the BaP exposure in the older groups (45-65) as compared to the younger groups (25-44).

The high contribution of rice to the total intake was mostly due to the high daily consumption of this food item (see **Chapter 3**), meaning that no zero-consumption days could be withdrawn from the consumption database during the simulations. Pork and fish were the highest food item contributors. Hence, the high intake of BaP found in the Capital of the country (region 1), as shown previously, can be explained by the high meat consumption profile in this region.

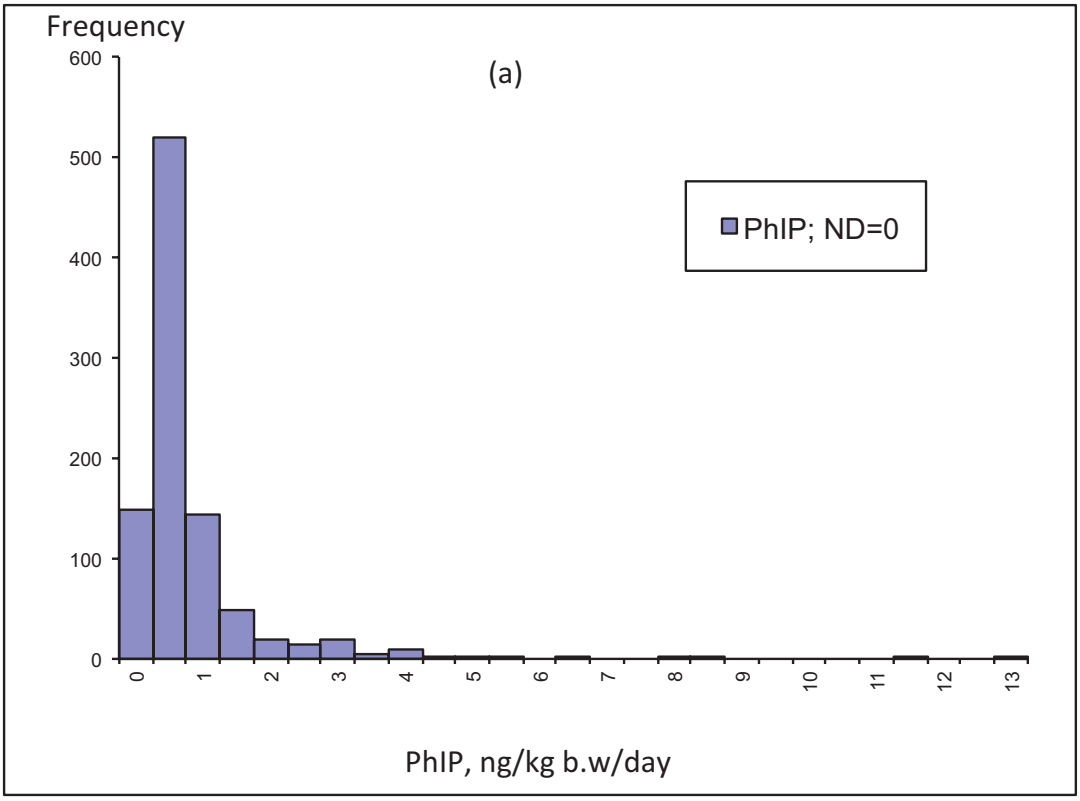
3.2. Exposure assessment to HAs

3.2.1. Deterministic approach

The exposure assessment to HAs was applied with the deterministic approach base on the occurrence concentration data that available in the literature review. A total of 240 red meat, poultry and fish items based on degree of doneness and cooking methods were identified from FFQ. One of the aims of this study was to quantify HAs formed in some meat/fish dishes cooked under regular household conditions. For this reason, the selected foods were processed following different cooking methods and conditions from the household previously described in **Chapter 3**. From the HAs content in cooked meat and fish (mean value) estimated based on literature data and from information on eating habits provided by the FFQ discussed above, three HAs (PhIP, MeIQx and DiMeIQx) daily intakes corresponding to the consumption of the studied meat and fish were calculated.

Mean and 95th percentile daily intakes assessed for these three HAs corresponding to the meat/fish products are given for the different population groups (gender, region, age group) in Tables 4.2, 4.3 and 4.4. Overall, the distributions of daily exposure to PhIP, MeIQx and DiMeIQx are presented in Figures 4.7 to 4.9 for the Cambodian population investigated in this work.

As previously explained in the material and methods section of this Chapter, two scenarios have been investigated: (i) scenario 1 where non detected values were replaced by zero (lower bound); (ii) scenario 2 where non detected values were replaced by LOD (upper bound). The mean daily intake for the whole group of adult population at the upper bound was 29 ng/person/day (0.5 ng/kg b.w/day), 15 ng/person/day (0.27 ng/kg b.w/day) and 3.7 ng/person/day (0.07 ng/kg b.w/day) for PhIP, MeIQx and DiMeIQx, respectively with the first scenario; values were 37 ng/person/day (0.7 ng/kg b.w/day), 16 ng/person/day (0.3 ng/kg b.w/day) and 5.4 ng/person/day (0.1 ng/kg b.w/day) for PhIP, MeIQx and DiMeIQx, respectively in the second scenario.



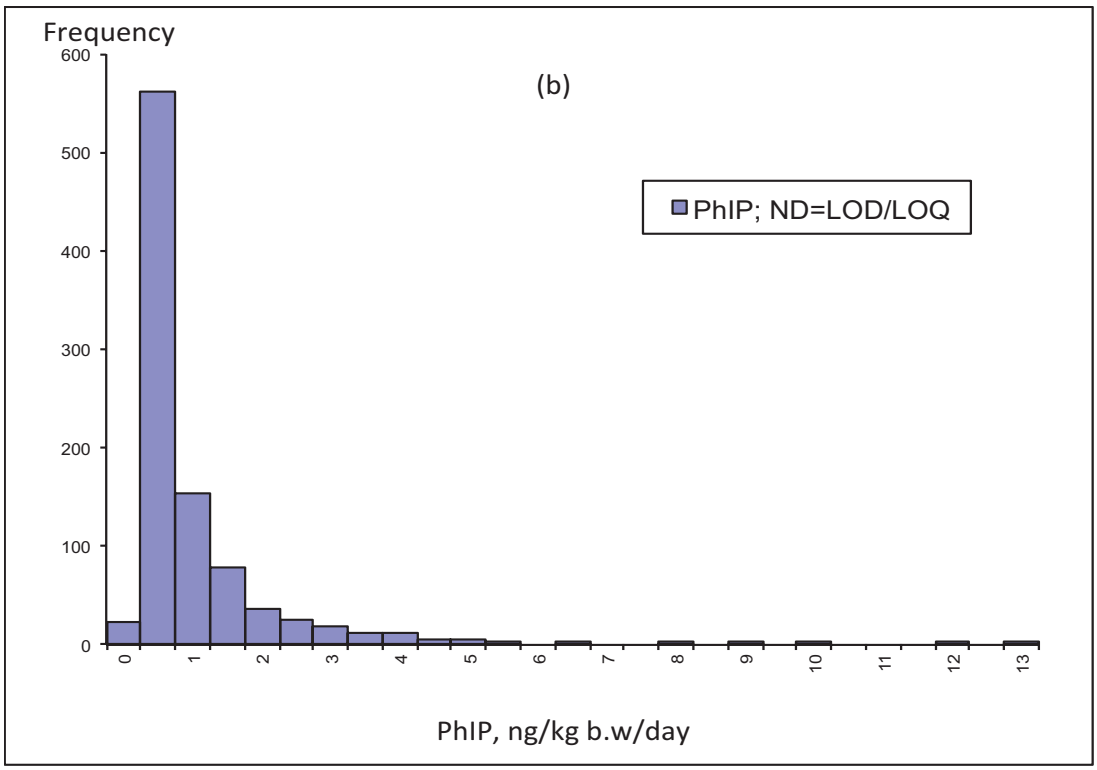
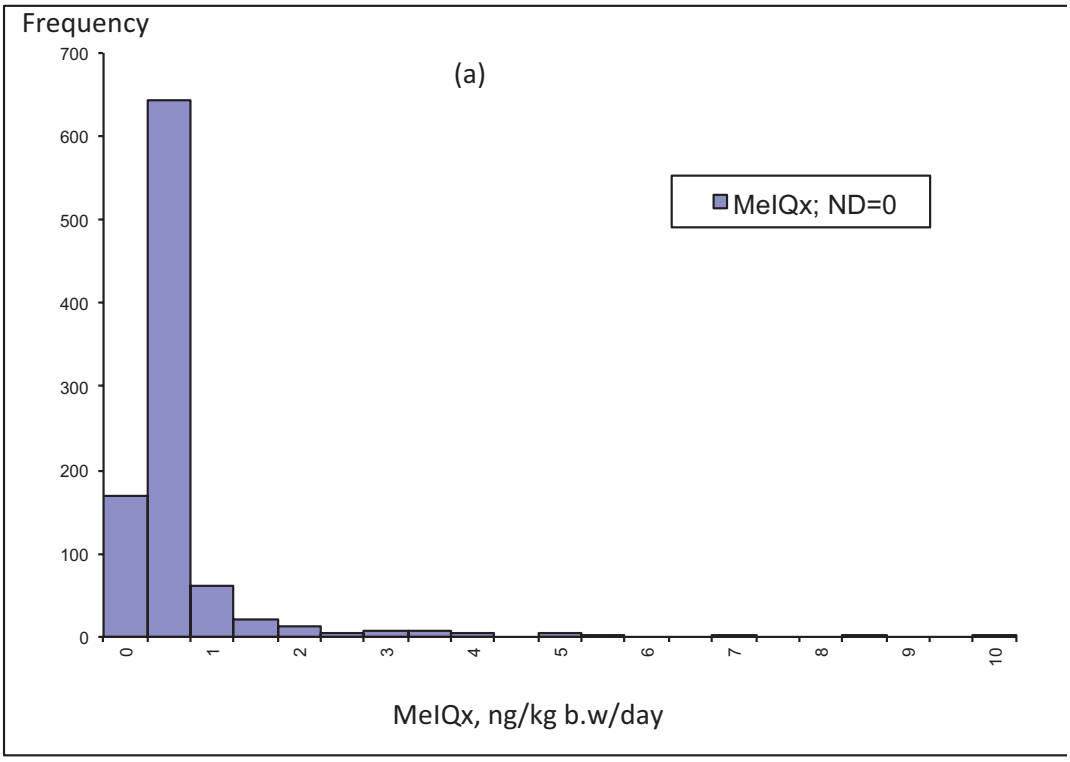


Figure 4.7. Distribution of PhIP daily intake (ng/kg bw/day) in a Cambodian population (n=941). (a) scenario 1 (ND = 0); (b) scenario 2 (ND = LOD)



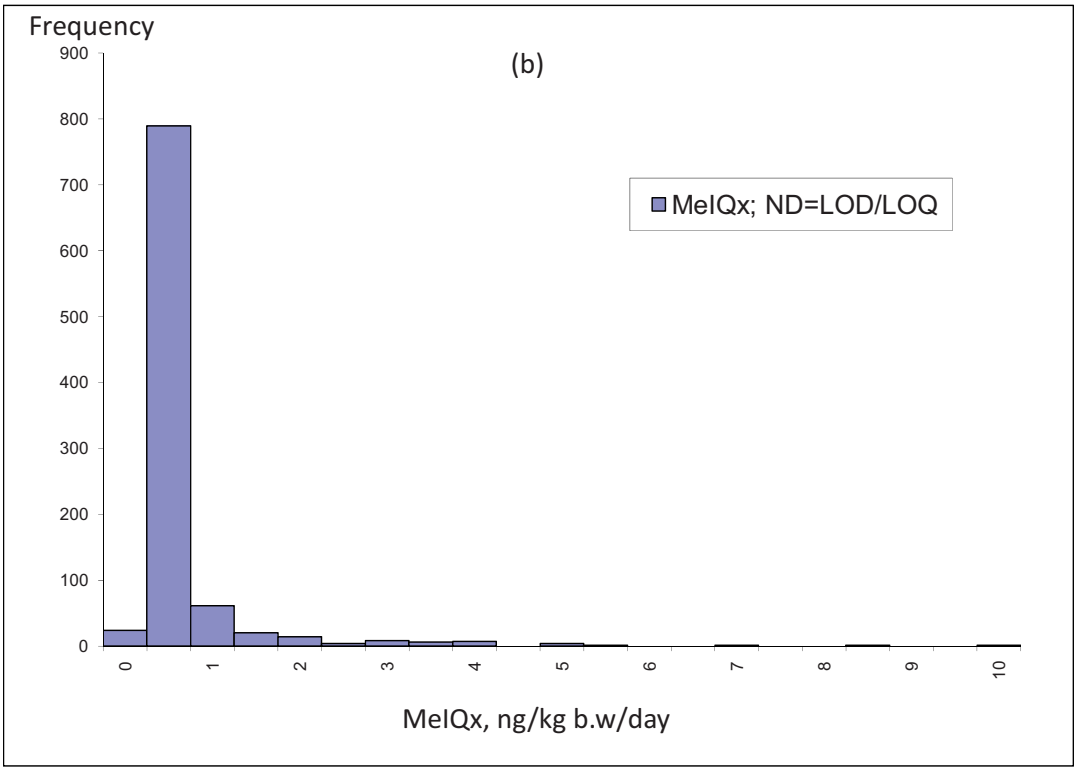
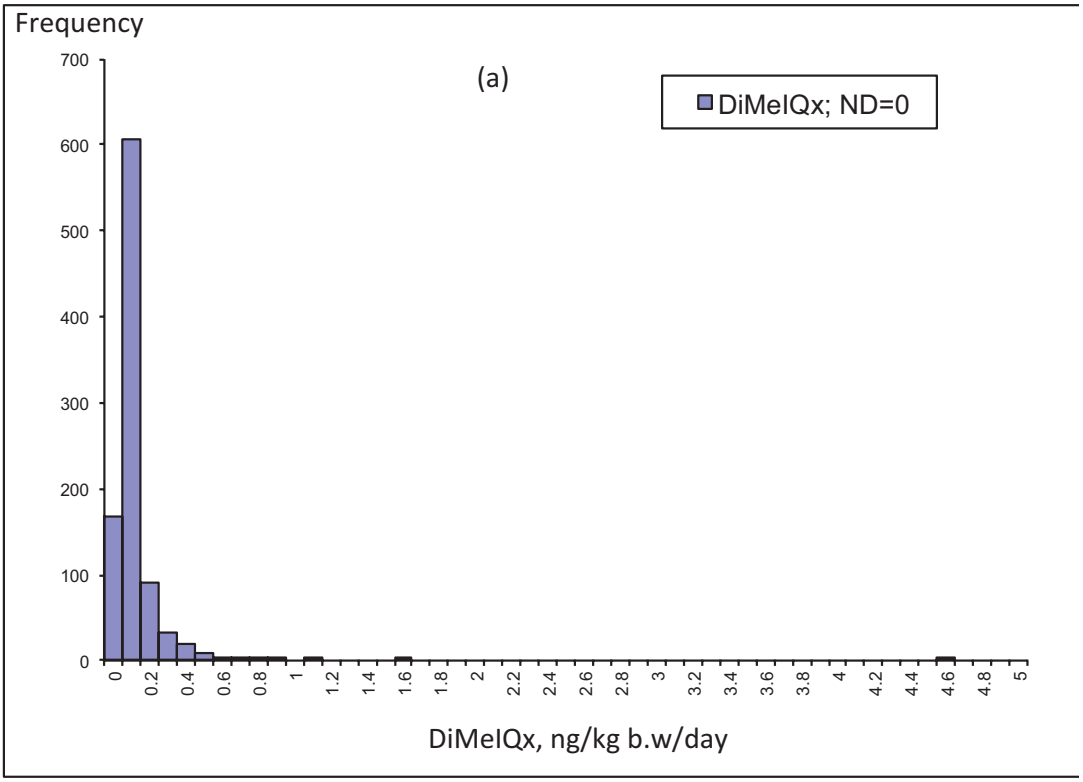


Figure 4.8. Distribution of MeIQx daily intake (ng/kg b.w/day) in a Cambodian population (n=941). (a) scenario 1 (ND = 0); (b) scenario 2 (ND = LOD).



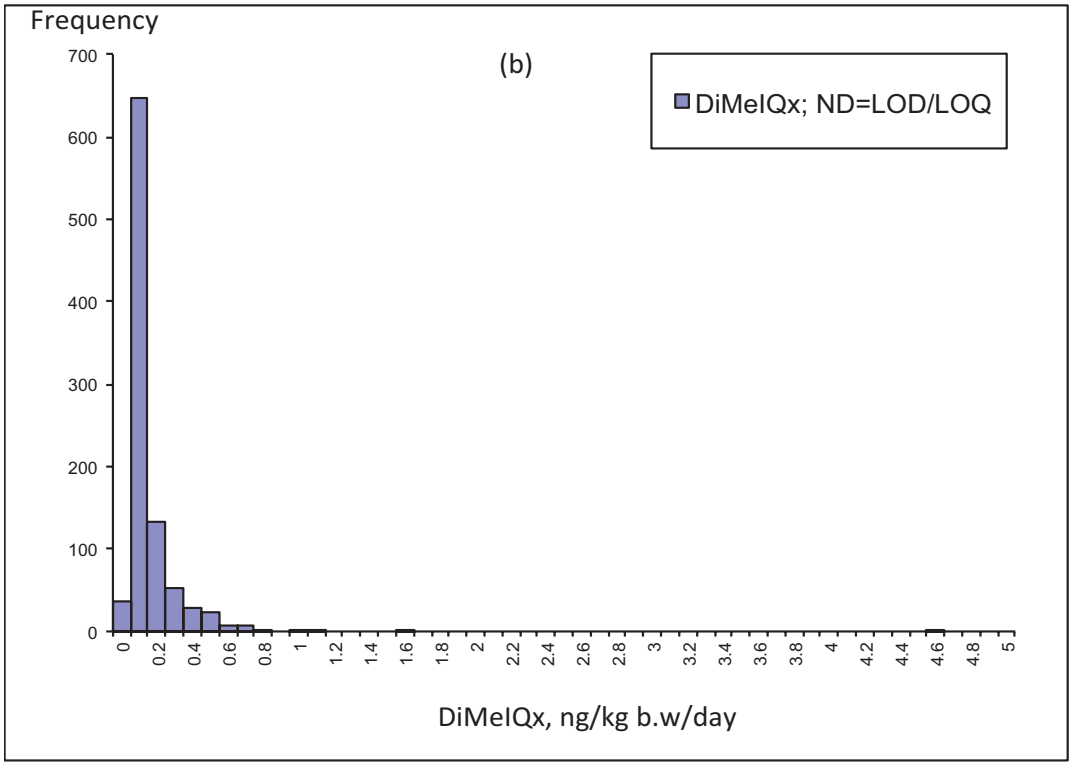


Figure 4.9. Distribution of DiMeIQx daily intake (ng/kg b.w/day) in a Cambodian population (n=941). (a) scenario 1 (ND = 0); (b) scenario 2 (ND = LOD).

It should be noted that a small part of the population had very high HAs intakes: the intakes at the 95th percentile were about three or four times higher than the mean intakes in the population (see Tables 4.2, 4.3 and 4.4). When looking at the gender, the daily mean exposure to the of PhIP, MeIQx and DiMeIQx was 40 ng/person/day (0.7 ng/b.w/day), 13 ng/person/day (0.23 ng/kg b.w/day) and 5 ng/person/day (0.08 ng/kg b.w/day) for men, respectively; and 38 ng/person/day (0.7 ng/kg b.w/day), 18 ng/person/day (0.3 ng/kg b.w/day) and 5.5 ng/person/day (0.1 ng/kg b.w/day) for women, respectively (scenario 2). The exposure to HAs was found statistically similar for men and women in both scenarios ($p > .05$).

PhIP contributed most (60%) to the total dietary HAs intake (estimated based on the three HAs considered here). The mean intake of PhIP was similar ($p >0.05$) in the dry and wet seasons (0.7 ng/kg b.w/day in scenario 2). On the opposite, there was a significant difference in the exposure to MeIQx ($p <0.05$) between the dry and wet seasons: the exposure of MeIQx was 50% higher in the wet season (0.4 ng/kg b.w in scenario 2) than during the dry season (0.2 ng/kg b.w/day in scenario 2). The mean intake of DiMeIQx was found to be higher in wet season than in dry season in both scenarios ($p >0.05$). The exposure of DiMeIQx of the studied population was 30% higher in the wet season (0.08 ng/kg b.w/day and 0.12 ng/kg b.w/day for scenario 1 and 2, respectively) than during dry season for both scenarios (0.05 ng/kg b.w/day and 0.09 ng/kg b.w/day for scenario 1 and 2 respetively).

The considered population was differently exposed to the HAs investigated depending on its location. In particular, the Capital population was exposed near three times more to PhIP, MeIQx and DiMeIQx than the populations from regions 2 and 4, and almost 1.5 times more to PhIP and DiMeIQx compared to the population from region 3. The highest exposure to the three HAs was found in region 1 (the Capital) and the lowest in region 2. A Kruskal-Wallis test showed that the exposure to these three HAs in region 1 had a statistically significant difference with those exposures in regions 2, 3 and 4 ($p <0.05$). For the Capital population, the highest intakes (95th percentile) of PhIP, MeIQx and DiMeIQx were 4.1, 2.48 and 0.6 ng/kg b.w/day, respectively (scenario 2).

Differences can also be noted with the age of the considered population. Hence, the highest intake of PhIP, MeIQx and DiMeIQx was found in the first age range (0.8, 0.4 and 0.12 ng/kg b.w/day in scenario 2, respectively), the second highly exposed group being the last age range. A statistical significant difference was noted between first age group with the second and the third age groups ($p <0.05$), but not with the last age group ($p >0.05$) for all studied regions.

Table 4.2. Dietary exposure to PhIP in a Cambodian population (n=941).

Dietary exposure to PhIP	Scenario 1 (ND = 0)					Scenario 2 (ND = LOD)				
	Mean ±SD	95th p	Mean ±SD	95th p	p-value[1]	Mean ±SD	95th p	Mean ±SD	95th p	p-value[1]
	ng/person/day		ng/kg b.w/day			ng/person/day		ng/kg b.w/day		
General population	28.6±53.9	126.4	0.53±1.1	2.6		37±61.4	177	0.7±1.2	2.9	
Men	36.6±59.8	184.7	0.5±0.9	2.7	0.2	40.3±63.6	198.6	0.7±1	2.9	0.2
Women	37.6±52.5	111.5	0.54±1.1	2.65		37.8±60.1	144.4	0.7±1.2	2.8	
Season										
Dry season	26.7±45.2	120.5	0.5±0.8	2.3	0.7	38.8±56.3	180	0.7±0.96	2.8	0.1
Wet season	30.6±62.3	137.9	0.6±1.3	2.7		37.4±66.7	174.4	0.7±1.4	2.9	
Region										
R1	52.6±78.3	214.9	0.96±1.5	4	0.001	65.2±83.8	234.8	1.2±1.6	4.1	0.001
R2	13.2±20.9	51	0.24±0.4	1		15.6±23.8	62.8	0.3±0.4	1.2	
R3	36.8±53.1	131.4	0.5±1.1	2.4		40.6±64.5	178.6	0.75±1.3	2.8	
R4	20±29.5	91.2	0.4±0.55	1.6		27.8±35.9	114	0.5±0.6	2.1	
Age range										
25-34[2]	34.3±61.3	156.6	0.66±1.2	2.8	0.001	43.8±66.1	191	0.8±1.3	3.4	0.000
35-44[2]	20.4±34.3	89	0.4±0.7	1.5		27±47	98	0.48±0.9	1.8	
45-54	23.9±51.8	119	0.4±1	1.8		34.3±59.3	151	0.6±1.1	2.7	
55-65[1]	32.9±57.5	185	0.6±0.96	2.8		45.2±67.4	214	0.8±1.1	3.6	

[1] *p-value obtained from Kruskal-Wallis test (for non-parametric data)*

[2] *p-value >0.05 between the first and the last age range (Scenario 1 and 2).*

Table 4.3. Dietary exposure to MeIQx in a Cambodian population (n=941).

Dietary exposure to MeIQx	**Scenario 1 (ND = 0)**					**Scenario 2 (ND = LOD)**				
	Mean ±SD	**95th p**	**Mean ±SD**	**95th p**	**p-value[1]**	**Mean ±SD**	**95th p**	**Mean ±SD**	**95th p**	**p-value[1]**
	ng/person/day		**ng/kg b.w/day**			**ng/person/day**		**ng/kg b.w/day**		
General population	15.2±43.5	96.2	0.27±0.8	1.4		16.1±43.4	76.6	0.3±0.8	1.5	
Men	12.4±37.3	69.8	0.2±0.7	1.2	0.2	13.4±37.2	70	0.23±0.67	1.2	0.1
Women	16.7±46.5	86	0.32±0.86	1.6		17.6±46.5	86.8	0.3±0.86	1.6	
Season										
Dry season	7.9±23.6	47.4	0.14±0.4	0.8	0.001	9.1±23.7	48.5	0.2±0.4	0.8	0.001
Wet season	23.4±57.2	128	0.4±1	2.5		24±57.2	129.4	0.4±1	2.5	
Region										
R1	28±54.6	129.3	0.5±1.1	2.5	0.000	29.2±54.4	129.3	0.5±1.1	2.48	0.000
R2	2.5±9.3	7.5	0.05±0.16	0.14		3±9.5	9.3	0.05±0.16	0.17	
R3	18.6±51	112	0.3±0.9	2.2		19.7±51	113.6	0.4±0.9	2.3	
R4	9±33.8	32.6	0.16±0.6	0.5		9.9±34	34	0.17±0.56	0.56	
Age range										
25-34	19.8±50	107.8	0.38±1	2.3	0.001	20.7±49.9	110	0.4±1	2.3	0.002
35-44	11±29.8	61.2	0.2±0.5	1.1		11.8±30	62	0.2±0.5	1.1	
45-54	13.6±49.2	64.9	0.2±0.8	1.2		14.5±49	65	0.2±0.8	1.2	
55-65	11±28.8	54	0.2±0.5	0.9		12.2±28.8	54.2	0.2±0.5	0.9	

[1]*p-value obtained from* Kruskal-Wallis test (for non-parametric)

Table 4.4.. Dietary exposure to DiMeIQx in a Cambodian population (n = 941).

Dietary exposure to DiMeIQx	Scenario 1 (ND = 0)					Scenario 2 (ND = LOD)				
	Mean ±SD	95th p	Mean ±SD	95th p	p-value[1]	Mean ±SD	95th p	Mean ±SD	95th p	p-value[1]
	ng/person/day		ng/kg b.w/day			ng/person/day		ng/kg b.w/day		
General population	3.7±9	15	0.07±0.2	0.3		5.4±10	20.8	0.1±0.2	0.4	
Men	3.4±6.5	15.2	0.06±0.1	0.25	0.03	5±7.5	19.8	0.08±0.12	0.3	0.05
Women	3.9±10.2	15.4	0.08±0.2	0.3		5.5±11	21.2	0.1±0.2	0.4	
Season										
Dry season	2.8±4.9	11.7	0.05±0.08	0.2	0.2	4.6±6.3	17.2	0.08±0.1	0.34	0.2
Wet season	4.7±12	18.3	0.09±0.26	0.4		6.2±12.8	24.8	0.12±0.3	0.4	
Region										
R1	7.8±15.5	27	0.14±0.33	0.49	0.000	9.9±15.9	32.1	0.18±0.3	0.6	0.000
R2	1.2±2.2	5	0.02±0.04	0.08		1.8±2.6	6.6	0.03±0.05	0.1	
R3	3.3±5.7	15	0.06±0.1	0.3		5.5±7.7	20.6	0.1±0.14	0.4	
R4	2.3±4	8.7	0.04±0.07	0.16		3.8±5.3	12.4	0.07±0.09	0.2	
Age range										
25-34[2]	4.6±11.6	17.6	0.09±0.26	0.3	0.001	6.4±12.2	23.2	0.12±0.27	0.4	0.03
35-44	3±7.7	11.4	0.05±0.14	0.2		4.2±8.5	15.7	0.07±0.15	0.27	
45-54	2.9±5.7	15.7	0.05±0.1	0.26		4.7±7.4	21.7	0.08±0.12	0.35	
55-65[2]	3.5±6.5	15.7	0.06±0.1	0.28		5.3±7.3	24	0.09±0.12	0.34	

[1]p-value obtained from Kruskal-Wallis test (for non-parametric), [2] p=0.1 between the first and the last age range for the second scenario

3.2.2. Cooking practices and preference of intake

As detailed previously, only meat/fish products were considered for assesssing exposure to HAs, as these food categories have been reported in the literature as the main food contributors for such contaminants. So, in this work we have paid attention to the cooking practices used by the Cambodian population investigated, as such practices are well-known to affect the formation and the levels of these neoformed contaminants. Overall, the mean daily consumption of pork, beef, poultry and fish prepared by different cooking methods are presented in **Figure 4.10**.

Fresh or marinated meat (pork, beef and poultry) and fish were the most common types of food for both sexes. The mean intake of grilled and pan-fried fish was 24.7 and 21.3 g/person/day, respectively; the average consumption of grilled and pan-fried pork was 11.9 and 13.7 g/person/day, respectively.

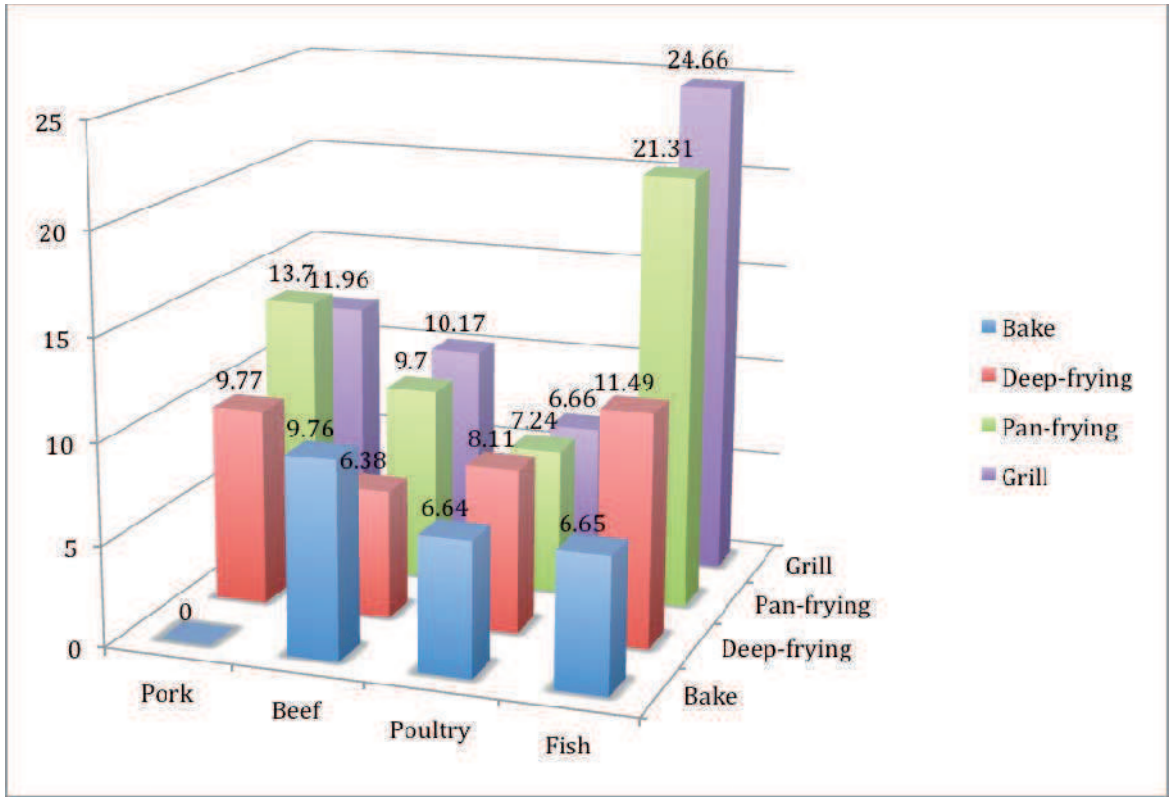


Figure 4.10. Mean daily consumption of meat/fish prepared with different cooking methods for the Cambodian population studied.

Table 4.5 and **Table 4.6** gather the mean daily intake per person (g/day) (along with standard deviation) for meat/fish cooked by four cooking methods under high temperature, and their contribution within regions and age groups. The mean intakes of grilled and pan-fried red meat (pork and beef) and poultry were higher in the Capital ($p < 0.05$) compared to the other regions. The similarity of fish intake in terms of the high cooking temperature (grill, pan-frying and deep-frying) was found in all regions ($p > 0.05$).

Table 4.5. Meat/fish daily consumption for the investigated Cambodian population (n=941), depending on its location.

Food items	**Food consumption (ng/person/day)**			
	Region 1	**Region 2**	**Region 3**	**Region 4**
	Mean ±SD	**Mean ±SD**	**Mean ±SD**	**Mean ±SD**
Grilled[1]				
Pork	15.2±18.8	10.6±14.2	10.7±13	11±14
Beef	13±18	12±18	8.4±10	7.4±10
Poultry	10.5±15	4.2±5	5±8.6	5±7.6
Fish	22.8±24.9	26.5±24.8	26±25	22±21
Sausage	6±8.5	5±7.4	4.8±8.5	5.5±7.3
Pan-fried[1]				
Pork	15.2±18.8	10.6±14.2	10.7±13	11±14
Beef	11±12.8	12±15	9±17	7.6±10.4
Poultry	10.4±23	6.2±11.8	5.5±8.7	5.6±8.5
Fish	20.9±21.5	20.7±18.6	22.7±23.7	20.5±20.3
Sausage	4.6±5.6	3±4	4.4±5.5	5±9
Deep-fried				
Poultry	10.2±19	7.6±10.8	4.9±5.6	10.3±12.3
Fish	11.4±14.3	12.8±12.6	11.2±15.4	10.5±15.3
Baked				
Poultry	8.8±17.9	4.7±5.7	5.5±8.6	5.6±10.3
Fish	5.4±8.9	8.4±13.5	7.6±13	9.6±9.4

p-value obtained from Kruskal-Wallis test (for non-parametric data)

[1]*p-value <0.05: between region 1 and the other regions for grilling and pan-frying of pork, beef and poultry.*

Difference in fish intake between age groups was found with the youngest age-range group consuming less fish (grill and deep-frying) than the other age groups ($p < 0.05$). For grilled and pan-fried beef, the first age group consumed more than the third and the last age group ($p < 0.05$) but less than the second age group ($p < 0.05$). Poultry consumption was found similar between age groups in terms of grilled and pan-fried ($p > 0.05$), except that the last age group consumed more pan-fried poultry than the other age groups ($p < 0.05$), and the first age group consumed less grilled poultry ($p < 0.05$) compared to the other age groups.

Table 4.6. Meat/fish daily consumption for the investigated Cambodian population (n=941), depending on its age range.

Food items	**Food consumption (ng/person/day)**			
	Age-range 25-34	**Age-range 35-44**	**Age-range 45-54**	**Age-range 55-65**
	Mean ±SD	**Mean ±SD**	**Mean ±SD**	**Mean ±SD**
Grilled[1]				
Pork	13.2±14.3	12±15	9±11	13.4±22.2
Beef	10.7±14.6	9±10.6	12±19	6.7±8.7
Poultry	7.9±14	6±8.9	6.2±7.6	4.7±6.7
Fish	22±21.6	25.8±25.7	26.6±28	26.5±22.3
Sausage	6±8	5±11	4±6	3.6±5.3
Pan-fried[1]				
Pork	14±16	12.2±14.7	13.6±17.4	14.7±17.4
Beef	9.6±14.4	9.4±12.1	10.6±15.8	9±10.2
Poultry	7.5±11.6	7±11.7	5.8±6.9	9.3±34.8
Fish	21±21	21±18	20±21	24±26
Sausage	5±7	4±4.6	4.4±6.6	2±2.2
Deep-fried[1]				
Poultry	9.3±16.4	8±11.4	4±4	11±12
Fish	9.6±12.3	15.3±17.6	10.7±12.3	12.3±20
Baked				
Poultry	7.8±16	5±6	5±6	4±7
Fish	4.6±6.8	12.7±16.6	7.2±7.8	1.4±0.5

p-value obtained from Kruskal-Wallis test (for non-parametric); [1]p-value <0.05: between first age range and the other age range for grill and pan-frying and deep-frying of fish.

The difference in grilled sausage intake was found significant between all age ranges ($p < 0.05$), the first age range being a higher consumer. Similarities were found for pan-fried sausage for the first, second and third age range ($p > 0.05$). The lowest consumption of sausage was found in the last age range ($p < 0.05$) compared to the other age ranges.

On the whole, among the four cooking methods at high temperature (grilling, pan-frying, deep-frying and baking), grilling and pan-frying were found to be the preferred ones for the studied Cambodian population. **Figure 4.11** presents the frequency of consumers (among the studied Cambodia population) using the four cooking methods. In this study, when the Cambodian people consumed red meat (marinated or fresh meat) they used more pan-frying for pork and grilling for beef. Among the four cooking method, fish and poultry were more consumed grilled and pan-fried. However part of fish was also deep-fried; it can be noted that, for this cooking method, fish was the preferred food cooked. In contrast, baking was mainly applied for poultry (mostly chicken in practice), to a lower extent for fish (17.9 and 5.8% of consumers, respectively), and to the lowest extent for red meat.

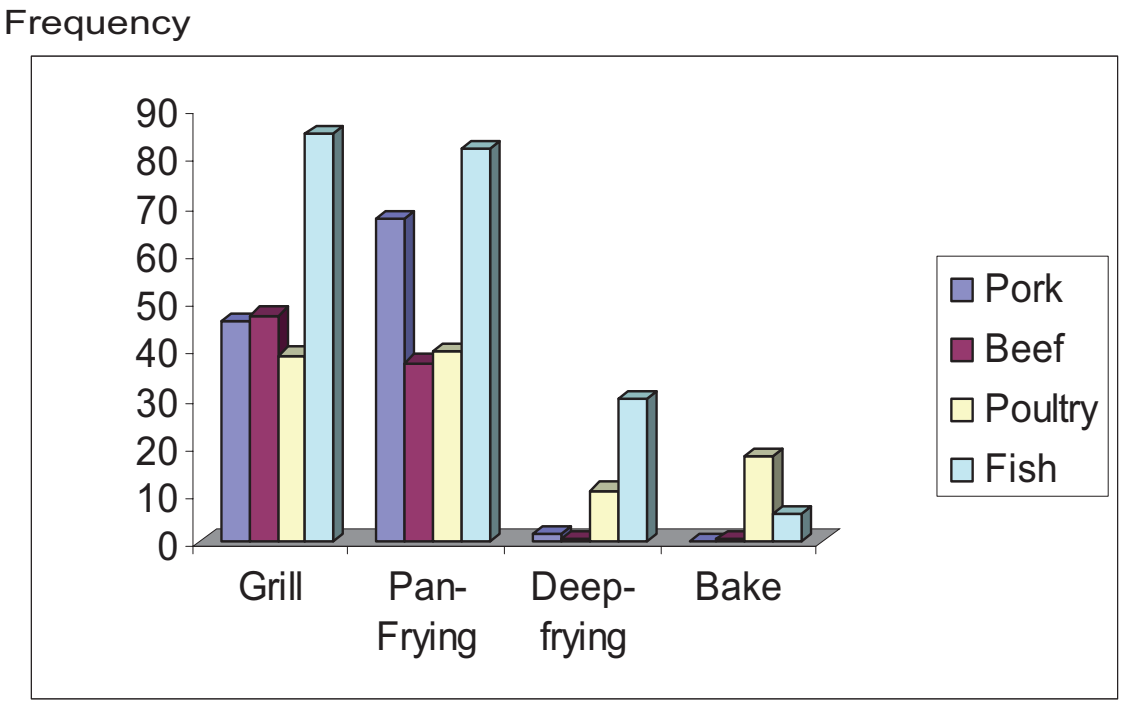


Figure 4.11. The frequency (%) of cooking methods for pork, beef, poultry and fish.

Figure 4.12 depicts the consumption frequency of grilled and pan-fried meat/fish among the population investigated. In Cambodia, around 54% of studied population consumed grilled and fried

red meat at least once a week, and 43% once a month. Poultry was consumed once a month by 71% of the studied population, while 13.8% of the population consumed grilled and pan-fried poultry at least once a week. Fish was consumed at least once a week either grilled or pan-fried by 65% of the studied Cambodian population and once a month by 31%.

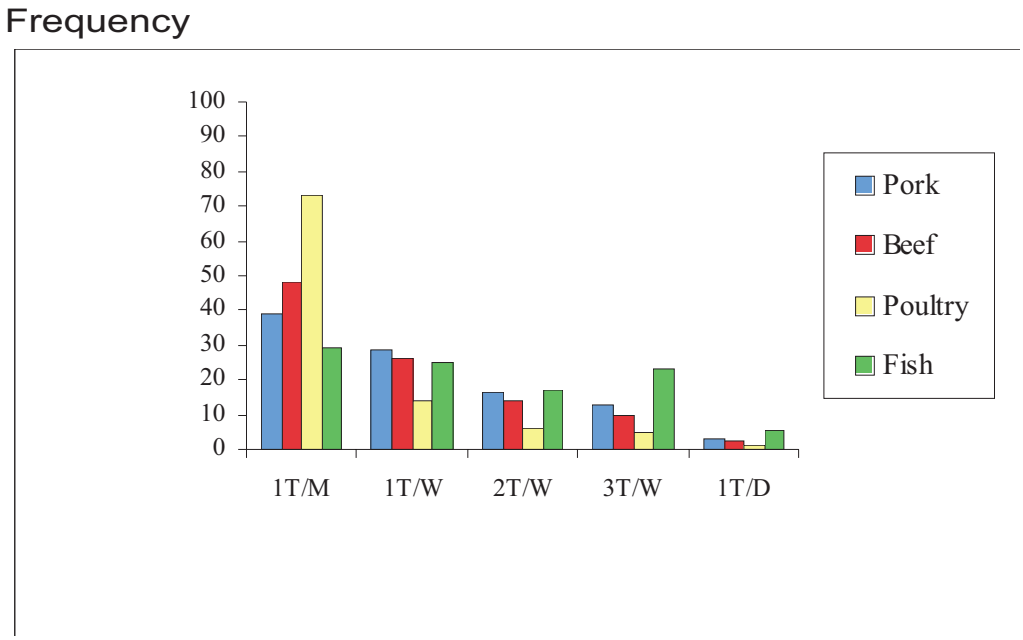


Figure 4.12. Consumption habits for the Cambodian population studied, relative to grilled and pan-fried meat/fish (expressed in % of studied subjects).

3.2.3. Cooking practices contributing the most to the HA*s* intake

Figure 4.13, 4.14 and 4.15 show the meat, poultry and fish (fresh and marinated) contribution to the total intake of PhIP, MeIQx and DiMeIQx to the study population. Pork (8.4% for fresh pork and 48% for marinated pork) was the most contributor to the PhIP intake in the study populations, fresh fish (47%) was the most contributor to the MeIQx, follow by pork (23% which 14% for fresh pork and 9% for marinated pork) and The major contributors to DiMeIQx were marinated pork (27%) and marinated beef (17%).

The well done pork meat products category is the main contributor to the average daily intake of PhIP and MeIQx of adult Cambodia in the studied population (**see Fig 4.16 and 4.17**). For DiMeIQx, the well done pork and well chicken (**Fig 4.18**) were the principle contributors (26% and 23%,

respectively). In contrast, amount the potential source of the three HAs, very well done fish was the major contributor, in which, 21% for PhIP, 49% for MeIQx and 16% for DiMeIQx, respectively and follow by the very well done of beef (8%, 10% and 17% for PhIP, MeIQx and DiMeIQx, respectively).

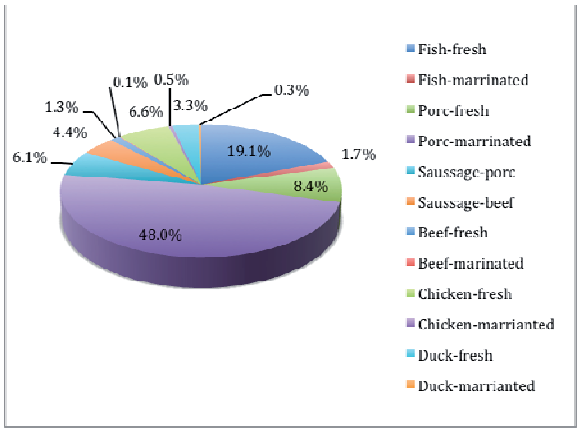


Figure 4.13. Contribution (%) of meat, poultry and fish to PhIP intake.

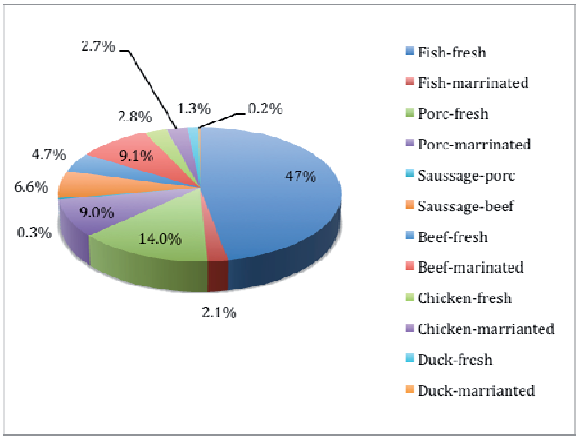


Figure 4.14. Contribution of meat, poultry and fish to MeIQx intake.

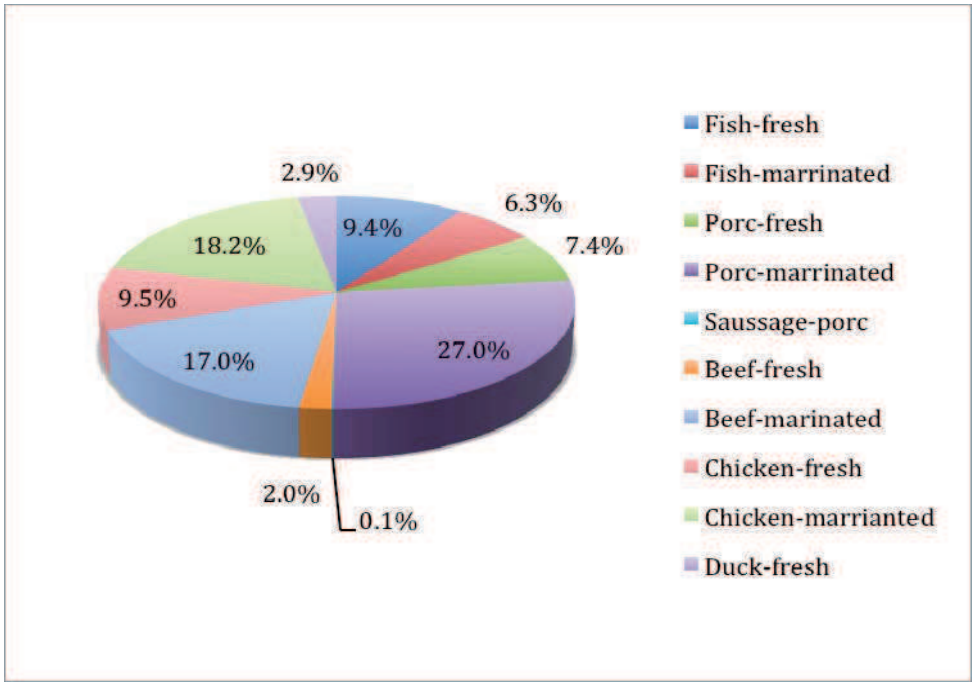


Figure 4.15. Contribution of meat, poultry and fish to DiMeIQx intake.

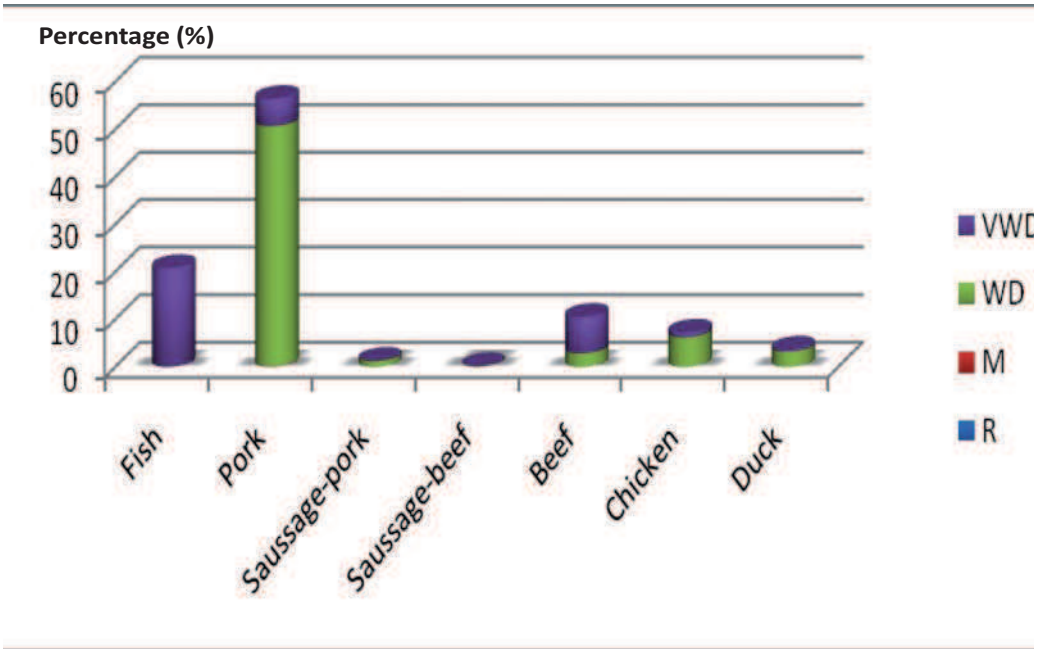


Figure 4. 16. The contribution (%) of meat, poultry and fish with difference degree of doneness level to PhIP intake.

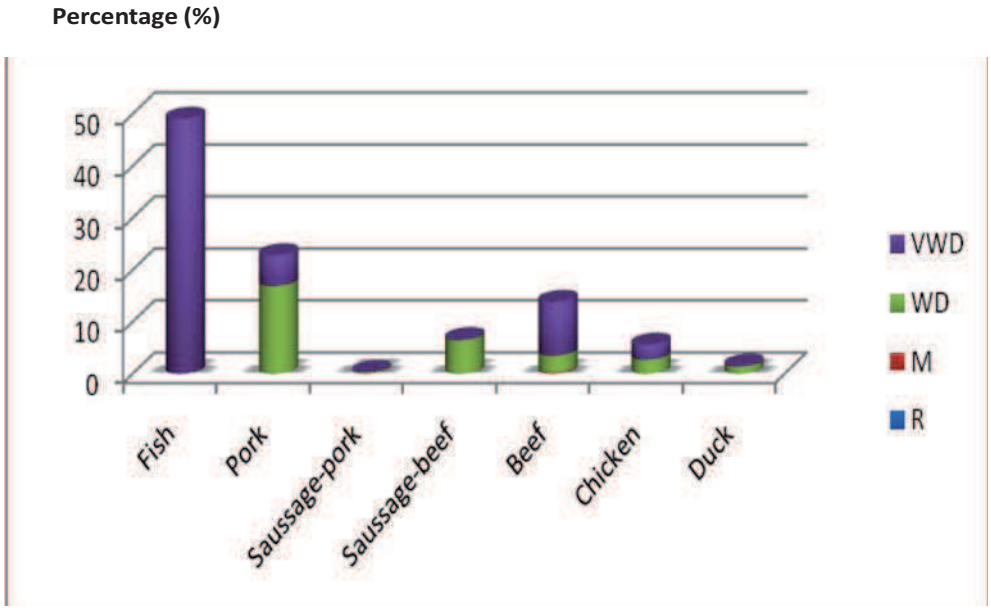


Figure 4. 17. The contribution (%) of meat, poultry and fish with difference degree of doneness level to MeIQx intake.

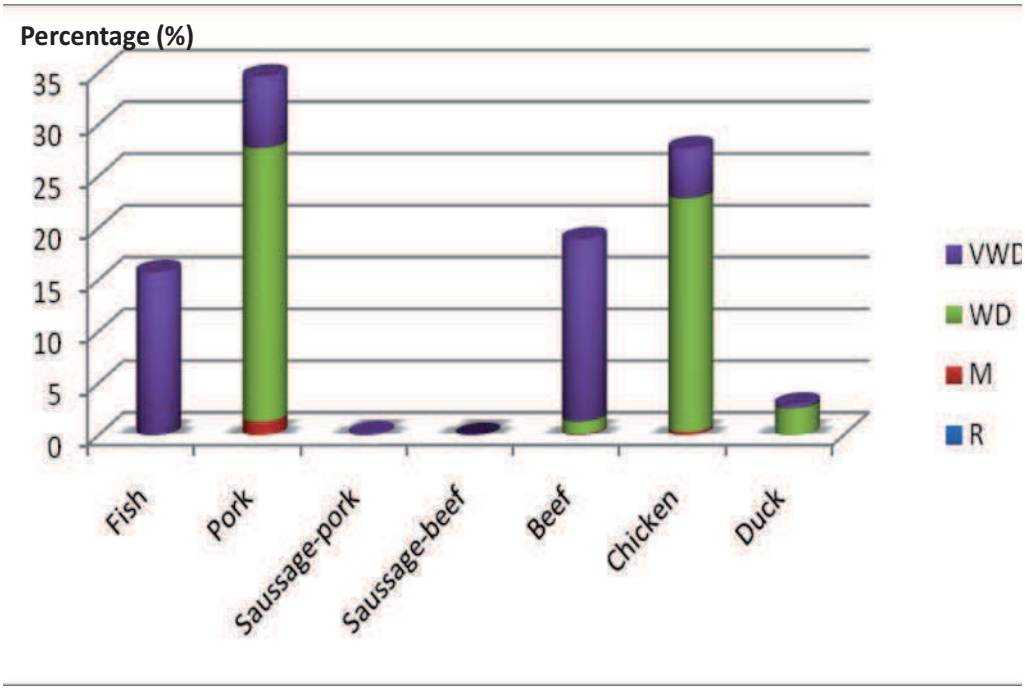


Figure.4. 18. The contribution (%) of meat, poultry and fish with difference degree of doneness level to DiMeIQx intake.

Our results show that in general pan-frying or grilling of meat, poultry and fish provided the higher contribution to the total intake of HAs as compared to deep-frying and baking (**Fig 4.19, 4.20 and 4.21**). The same percentage of contribution of grilling and pan-frying (48%) of meat, poultry and fish was found for the PhIP dietary intake. In which pan-frying pork provided about 44%, grill fish (18%), grill pork (11%) and grill beef (10%). For MeIQx, pan-frying of meat and fish was the most contributor (56% in which grill-fish was 29% and grill-pork 15%), followed by grilling (28%). The range of contributor was found for DiMeIQx, where pan-frying of meat and fish provided 52% (grill-pork contributed 31%) and 43% of grill-meat and fish (17%, beef, 11% chicken, 8% fish).

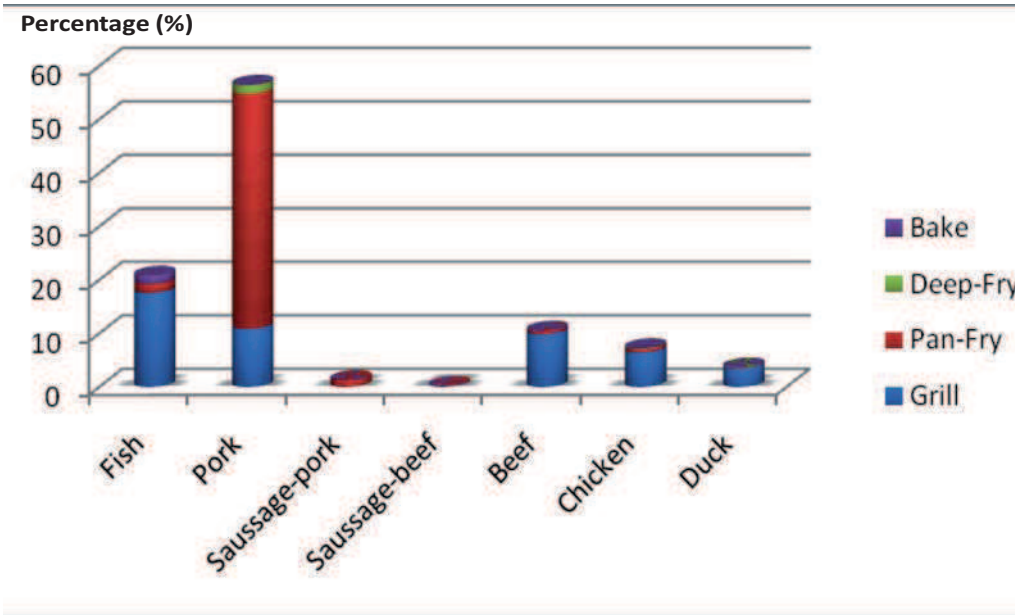


Figure. 4. 19. The contribution (%) of meat, poultry and fish with difference degree of doneness level to PhIP intake.

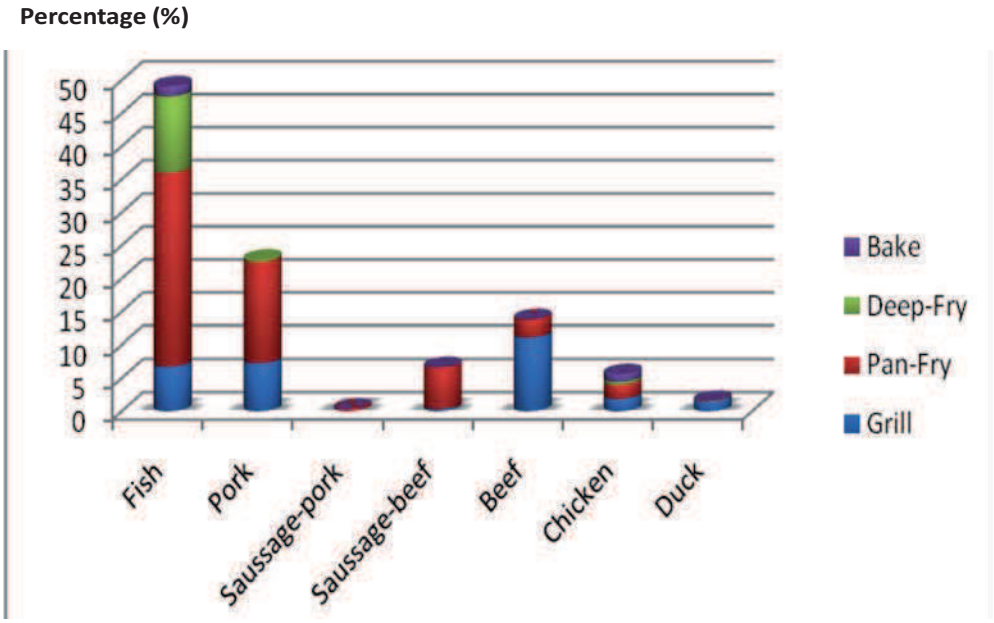


Figure 4. 20. The contribution (%) of meat, poultry and fish with difference degree of doneness level to MeIQx intake.

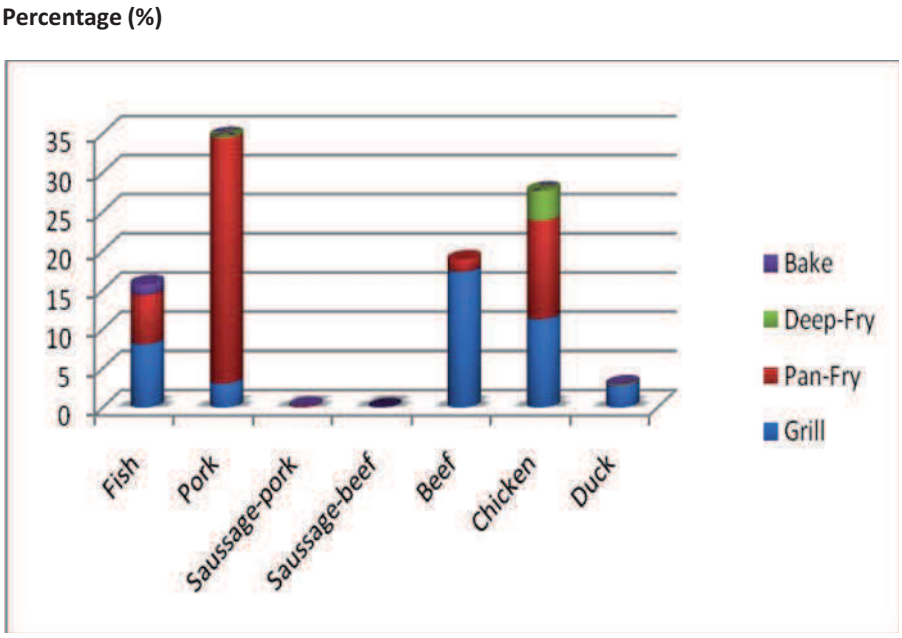


Figure.4. 21. The contribution (%) of meat, poultry and fish with difference degree of doneness level to DiMeIQx intake.

4. Discussion

To our knowledge, this work is the first study that describes the meat/fish consumption and cooking methods used in the Cambodian population. The sources of food consumption data used in this study were the most updated individual food consumption survey conducted in Cambodia, and the only food survey available at a national level. Individual food consumption data are the most relevant sources to evaluate food consumption of the studied population (25-65 years old) and cover both foods consumed at home and outside. In addition, information on cooking method was completed. The consumption data obtained from this survey data can estimate real consumption levels at national level in both urban and rural areas. On the other hand, food descriptors are provided in terms of information about method of cooking and degree of doneness, which highly correlate with the formation of HAs and BaP in meat and fish.

4.1. Discussion relative to the dietary exposure to BaP

As already mentioned, the main source of exposure to PAHs for non smoker adults is food, which contributed to more than 90% of total exposure (SCF, 2002; WHO, 1998). Dietary intake of food contaminants depends on both the nutritional habits of the examined population group and the concentrations of contaminants in food. Food consumption data from the national individual food consumption survey were used in the calculation of average intake for the general population. Data include the size of the population and average amounts of daily consumed food items in Cambodia.

The main results about exposure issued from the assessments of BaP in this study (55.6 ng/person/day) showed that the Cambodian population exposure to BaP is lower than in other Asian countries, such as China (571.6 and 487.6 ng/person/day for men and women respectively) (Xia *et al.*, 2010) and Korea (169.4 ng/person/day) (Lee *et al.*, 2007; Yoon *et al.*, 2007) as well as in European countries such as Spain (890 ng/person/day for men and 730 ng/person/day for women or 10 ng/kg b.w/day for both sexes) (Martí-Cid *et al* ., 2008; Martorell *et al.*, 2010) and France where the mean intake of BaP from food was about 245 ng/person/day (4 ng/kg b.w/day, assuming a body weight of 60 kg) (EFSA, 2008). Kazerouni *et al.* (2001) analysed 200 food items and estimated the intake of BaP for the population of Washington, D.C; the intake levels of this pollutant ranged from 400 to 1,800 ng/person/day. The estimated BaP daily intakes the population of Catalonia in Spain was 610–1,020 ng/person/day. The notable differences in dietetic habits for different countries could explain the differences among studies (De Vos *et al.*, 1990; Kazerouni *et al.*, 2001; Phillips, 1999).

Dietary exposure to BaP was found to be higher in women than men ($p<0.05$) (1.04 ng/kg b.w/day and 0.96 ng/kg b.w/day, respectively); this finding differs from other studies in which dietary exposure of females was lower than that of males (Martorell *et al.*, 2010). The high intake of BaP in the women population could be due to higher intakes of fat and oil and vegetables in the female group as compared with males as described in detail in chapter 3. As presented in figure 4.3, vegetables (27.35%) and fat and oil (17.9%) were the main contributors to BaP intake by the study population. Another striking point in our work is the BaP intake decrease as the age increased: from 58.5 to 53.5 ng/person/day between the 25-34 age range and the age range 55-65, for both males and females.

The variations in food habits between genders and age groups could explain the differences reported here. In the Cambodian population investigated, men consumed usually more meat and cereals (especially rice) than women and, in contrast, women consumed more vegetables and oil than men

(see **Chapter 3**); cereals, meat, vegetables, oil and fat are the food groups where the highest concentrations of PAHs were found (Cucó *et al.*, 2001; Dennis *et al.*, 1983; Falcó et al., 2003; Martí-Cid *et al.*, 2008; Xia *et al.*, 2010), which could explain the difference BaP exposure between gender and age groups.

Another important reason for the younger age to be more exposed to BaP than the older age was the significantly larger amount of ingested foods ($p < 0.05$). The young adult population (the first age-range group) consumed more meat than the other age groups, especially the older (mean meat consumption of 86.2 and 58 g/person/day respectively). Also, the first age-range group consumed more rice (i.e. 894 g/person/day) than the other age groups (i.e. 743.7-819 g/person/day). Finally, it appears that the major food contributors to the BaP exposure were the meat and cereal food groups for young adults (25-34).

Regarding the four studied regions, the exposure to BaP in the Capital (region 1) was the highest and differed significantly compared to the other regions. There were obvious differences in daily food consumptions between the Capital and the other three regions (see **Chapter 3**): in the Capital, meat and meat products were the most consumed, and they were found to be the food categories the most contaminated by BaP (Lee et al., 2007; Martí-Cid *et al.*, 2008). Overall, rice and meat were the second and the third contributors to the total exposure of BaP in this study. This finding is similar to other studies which also reported that meat and cereal products were the major contributors to the dietary exposure to BaP (Dennis *et al.*, 1983; Martorell *et al.*, 2010; Xia *et al.*, 2010).

Several studies have been carried out to determine the level of BaP intake associated with a normal, or average, human diet, and what the major sources of exposure contamination are. The cooking process is the most important factor in the formation of BaP in food, especially in meat (Gunter *et al.*, 2005; Kazerouni *et al.*, 2001). In general animal food products showed high contamination levels of BaP (Lee *et al.*, 2007), whereas vegetable food products showed relatively lower or higher levels of BaP depending on the potential contamination of the production areas (Li et al., 2008; Phillips *et al.*, 1999; Tuteja *et al.*, 2011; Xia *et al.*, 2010). Leaf vegetables are found to be more contaminated with PAHs as compared to underground vegetables, due to atmospheric deposition of these contaminants on vegetables (Tuteja *et al.*, 2011; Wickstrom *et al.*, 1986). In our study, we found that leaf vegetable contributed more to BaP exposure than the other vegetables (34.8%). In some studies the major dietary sources of PAHs were cereals and vegetables, rather than meat, except where there is a high consumption of meat cooked over an open flame (Phillips *et al.*, 1999; Dennis *et al.*, 1991; Ciemniak *et al.*, 2010). However, according to several studies, the major dietary contributors are

processing of foods at high temperatures, especially meat and meat products which represent the major source of human exposure to BaP (Ciemniak *et al.*, 2010; Dennis *et al.* 1983; de Vos *et al.* 1990; Tao *et al.* 2006). Grilled or barbecued meat caused a significant proportion (21%) of the average total daily BaP intake in USA according to the data obtained by Kazerouni *et al.* (2001); similar finding was reported in Estonia where the highest PAHs concentrations were detected in home-grilled pork samples (Kazerouni *et al.*, 2001; Reinik *et al.*, 2007). In the UK diet, it was found that the major contribution to PAHs exposure came from cereals (about one-third) and from oils and fats (another one-third); fruits, vegetables and sugars contributed much to the remainder contribution, while the contributions of meat, fish, milk and beverages were comparatively minor (Dennis *et al.*, 1983). However, it should be noted that, owing to the unpredictable summer weather in the UK, eating barbecued food is an infrequent activity for most of the population. In our study, meat was the second food contributor in BaP exposure, in which grilled pork (53%), beef (18%) and chicken (16%) were the most concerned. The estimated contribution of meat products to the overall intake of PAHs differs between countries from very low for UK, to 21% in USA, and 27% in France resulting in the second contributing food group after bread and cereals (SCF, 2002; EFSA, 2008; SCOOP, 2004).

The number of international reports concerning dietary exposure to PAHs as well as BaP for adult population is rather limited. However, dietary PAHs intake has been estimated in some European countries such as Austria, Italy, the Netherlands and the UK; furthermore estimated BaP intakes are also available for Germany, Sweden and USA (SCF, 2002; EFSA, 2008). In the 1980s and 1990s, the daily intakes of PAHs were estimated as follows for different countries populations: the UK 3.7 µg/person /day (Dennis *et al.*, 1983), the Netherlands 5–17 µg/person /day (de Vos *et al.*, 1990), Italy 3.0 µg/person /day (Lodovici *et al.*, 1995) and Greece 1.6–4.5 µg/person/day (only *via* vegetables) (Voutsa *et al.*, 1998). It must be taken into account that, in recent years, environmental PAHs concentrations have increased in all industrialized countries; consequently, the PAHs levels in foods are currently higher than they were 15–20 years ago (Xia et al., 2010). In relation to this, it was noted a remarkable increase in the current intake of PAHs in Spain: for a male adult living in Catalonia, the PAHs intake of the 2000 survey (i.e. 8.42 µg/person/day) is lower than the estimated ones reported in Catalonia in 2006 (i.e. 12.0 µg/person/day) (Falcó *et al.*, 2003; Martí-Cid *et al.*, 2008), which in turn are very similar to the PAHs intake recently reported by Ibáñez *et al.,* for a Spanish adult population (Ibáñez *et al.*, 2005). In that latter study, which included 40,690 individuals (35–64 years of age) from five regions of Spain (Catalonia not included), cereals (40.1%) followed by meat and meat products (20.9%), fish and shellfish (6.1%) were the food groups with the highest contributions to the total PAHs dietary intake (estimated as 8.6µg/person/day). Aquatic ecosystems are one of the

major sinks of PAHs, which tend to adsorb onto suspended particulate matter because of their lipophilic characteristics and to settle in the sediment (Shi *et al*., 2007). In the other recently study in Italy, BaP was found 66% of the aquaculture (Cirillo *et al*., 2009). The similar confirm was found in the study of the exposure assessment of Kuwaiti population, in which the concentrations of PAHs ranged between 30 and 247 ng/g dry weight. The mean exposures for the average adult Kuwaiti consumer to PAHs and BaP equivalents were estimated to be 1.3µg/day and 0.0013 µg/day, respectively (Alomirah *et al*., 2009).

In Cambodia, seafood was not the frequent food consumption due to their high prix and the unavailable in many regions in the countries, where the geography is far a ways from the sea (Chapter 3). In this study, seafood contributed about 8.4% of the total dietary intake of BaP.
In turn, Nwaneshiudu *et al.* determined the risks due to PAHs exposure from food consumption for the population of Azerbaijan; the most prevalent pathways of PAHs exposure from the dietary patterns of the Azerbaijani population were bread and bakery products, milk and dairy products, and egg products (Nwaneshiudu *et al.*, 2007). In a recent study, Reinik *et al.* determined the content of 12 PAHs in 322 meat products consumed by children and the general Estonian population (Reinik *et al.*, 2007); mean intakes by the general population from the consumption of meat products were 29 and 346 ng/person/day for BaP and total PAHs, respectively. Differences in estimated PAHs and/or BaP daily intake among studies and regions/countries can be explained by differences in dietary habits, culture, home cooking processing techniques, commercial processing techniques and contamination areas (EFSA, 2008; Houessou *et al*. 2007; Reinik *et al.*, 2007).

Due to different methods used for the estimation of intake of food contaminants and extensive variation of diets, results obtained in earlier studies differ to a large extent. Differences in estimated intake values are probably due to differences in the food groups/items as well as contaminants (BaP alone or other PAHs) considered, differences in analytical methods, and to statistical analysis of concentration values below the detection and determination limits (Reinik *et al.*, 2007). Analytical methods for BaP or PAHs analysis in food have been validated (Wenzl *et al*., 2006); in particular, the limit of detection that must be achieved for BaP is specified in Commission Regulation (EC) No 333/2007 to less than 0.3 µg/kg for those foodstuffs already covered by maximum levels. Most laboratories are able to comply with this limit; however, a few laboratories reported values that exceeded the target value by more than twofold (Wenzl, 2010).

In the current study, we utilized concentration data from different sources representing international studies performed over the past decade to determine those food products that contribute the most to

BaP exposure through ingestion for the Cambodian population. Due to the lack of food contamination data from Asian countries, only European countries were considered and used for this analysis.

Both SCF and the JECFA have concluded that a Toxic Equivalent Factor (TEF) approach to the risk assessment of PAHs was not appropriate due to limitations in the available data and because of different modes of action amongst different PAHs (SCF, 2002; FAO/WHO, 2005). They suggested to use BaP as a marker of occurrence and effect of the carcinogenic PAHs in food, based on examinations of PAHs profiles in food (Culp *et al.*, 1998; FAO/WHO, 2005). However, they recommended continuing collecting data on other PAHs in order to be able to evaluate the contamination of food commodities and any future change in the PAHs profile (Benford *et al*., 2010; SCF, 2002; EFSA, 2008).

In order to provide a likely intake of BaP covering the main food groups in the whole diet for the purposes of risk characterization, a separate determination of the range of intakes was conducted using only those studies that included foods from the range of major food groups. These studies included foods that were 'ready to eat' (e.g. cooked meat), and therefore included the likely concentrations of PAHs that arise due to cooking of food. From this analysis, mean intakes of BaP ranged from 1.4 to 420 ng/person/day. From this range, the JECFA selected the value of 240 ng/person/day (4 ng/kg bw/day assuming a body weight of 60 kg) as being representative of a mean intake for use in the risk characterization and assumed that high consumption could be 2 to 2.5 times higher, i.e. 8-10 ng/kg bw/day. Based on the margin of exposure (MOE) approach, the JECFA concluded that the estimated dietary intakes of PAHs were of low concern for human health. SCF also estimated a maximum daily intake of BaP from food of approximately 6-8 ng/kg bw/day for a person weighing 70 kg (SCF, 2002; Benford *et al*., 2010). The major foods reported to contribute to PAH exposure were consistently cereal products, due to high consumption, and oils, due to the both high contamination and consumption. Smoked foods also were generally reported to contribute significantly to national dietary exposure to PAHs (Benford *et al*., 2010).

With regards to exposure assessment of the Cambodian population, the estimated BaP daily intake (1.01 ng/kg b.w/day) was considerably lowest than the dose considered carcinogenic for experimental animals by SCF and JECFA (compared to the maximum value estimated by SCF and the JECFA). Based on this, the BaP exposure of the Cambodian population does not seem to be critical point for the action (estimated MOE: 87500) (SCF, 2002).

4.2. Discussion relative to the dietary exposure to HAs

This is the first study that describes the meat and fish preferred cooking methods and levels of HAs exposure in Cambodia through their daily diet, in order to compare their HAs exposure to that which was estimated for people in other parts of the world. Only three HAs have been investigated, chosen due to their food occurrence and toxicity.

The results of this study showed that for the three HAs considered, PhIP was the most contributor (37 ng/person/day, 60%) followed by MeIQx (16 ng/person/day, 27%) and DiMeIQx (5.4 ng/person/day, 9%). This finding was similar to the Singapore study, which also found PhIP as the largest contributor (29.06 ng/person/day, 58%), followed by MeIQx (12.65 ng/person/day, 25.3%) and DiMeIQx (5.89 ng/person/day, 11.8%) (Wong *et al.*, 2005). On the whole, the exposure to PhIP, MeIQx and DiMeIQx of the studied Cambodian population was lower when compared to the mean exposure reported in Europe (47.6, 29 and 14 ng/person/day for PhIP, MeIQx and DiMeIQx respectively) (Busquets *et al.*, 2004; Rohrmann *et al.*, 2007), New Zealand (72, 72 and 16 ng/person/day for PhIP, MeIQx and DiMeIQx respectively) (Thomson *et al.*, 1996) and in the USA (78.1 and 21.9 ng/person/day for PhIP and MeIQx respectively) (Cantwell *et al.*, 2004). As we observed, in general, the dietary intake is greatest for PhIP, followed by MeIQx, IQ and MeIQ.

PhIP is a genotoxic carcinogen that can cause cancers of the prostate, mammary gland and colon in rodents. Based on the exposure assessment of African-American males, 6 ng/kg b.w/day is thus a reasonable estimate for average PhIP exposure that can be used in calculating the margin of exposure (MOE) (Carthew *et al.*, 2010). The lowest MOE for colon tumours was 150,000 (BMDL10 values of 2.71 mg/kg/day for colon tumours). The exposure of PhIP (0.7 ng/kg b.w/day) in our study was lower compared to the value that Carthew et al. used for MOE calculation (6 ng/kg b.w/day). However, such a comparison is quite difficult, as the exposure assessment has been done only in several specific foods items due to the lack of contamination data.

In our work, exposure varied among individuals and among regions, since dietary preferences and variation in food preparation can greatly influence individual exposure. When stratified by region and age, the three HAs daily intake in the region 1 of our studied population was approximately 50% higher than in the other regions. By age groups, the daily dietary intake of HAs in the 25-34 years study population was approximately 50% higher than in the second and third age groups but similar with the last age group (55-65 years).

From the present study, it appears that middle-aged (25-34) or older (over 55) Cambodians generally consumed more meat and fish compared with other groups of respondents (Jahurul *et al.*, 2010). Meat cooked at high temperature is known to be the major source of HAs for human exposure; in the Cambodian diet, meat-based foods are commonly consumed in the Capital (see **Chapter 3**). A high consumption of meat cooked at high temperature (grilled, fried) is observed in the Capital (region 1). On the other hand, meat and fish cooked with grill or pan-fried which are prefered by Cambodian population, were the major food categories that contributed to the total intake of HAs in this studied populations (**Fig. 4.16 to Fig. 4.18**).

However, when compared to other countries, on average, Cambodian consumed about 75 g/person/day of all type of meats, which is less than reported for the other Asian, European and western countries. For instance, Wong *et al.* reported an average daily intake of 108.7 g/person/day for all meat types in Singapore (Wong *et al.*, 2005; Koh *et al.*, 2005); other population-based surveys have reported a daily consumption of meat from 80 to 160 g/person/day in the USA, Sweden, New Zealand and Japan (Augustsson *et al.* 1997; Byrne *et al.* 1998; Ferguson, 2002; Kobayashi *et al.* 2002). There were relatively few specific meat and fish dishes that accounted for a large contribution to this exposure (Jahurul *et al.*, 2010).

In this study, we found very little seasonal variation on exposure to HAs, especially to PhIP. As Cambodia is a tropical country, there is little seasonal variations in the type of food consumed (Wong *et al.*, 2005).

With regards to cooking methods, as frequently encountered in other Asian countries, the Cambodian population appears to consume a larger proportion of their meat boiled or steamed, and a smaller proportion fried, grilled or roasted compared with Western populations (Jahurul *et al.*, 2010; Koh *et al.*, 2005; Rohrmann *et al.*, 2002; Augustsson *et al.*, 1999). Among the meat/fish items and cooking methods considered, grilled and pan-fried fish and pork were the two most common types of food consumed by the studied population. Grilling and pan-frying were the most common cooking practices in this study for fish (85% grilled and 82% pan-fried), pork (50% for grilling and 67% for pan-frying) and beef (47% for grilling and 37% for pan-frying) (see Figure 4.8). Among all the study subjects combined, HAs intake by weight of pork, especially marinated pork (48%, 9% and 27% for PhIP, MeIQx and DiMeIQx, respectively) was the highest for all cooking methods, follow by fresh fish (19%, 47% and 9.4% fo PhIP, MeIQx and DiMeIQx, respectively) (see Figure 4.10, 4.11, 4.12).

A recent review describes the factors that affect the formation of HAs in foods, such as the cooking method, including temperature, time, and frequency of turning of meat, during cooking. Other factors depending on the type of food and the recipe followed are pH, amounts of HAs precursors, types of amino acids, presence of certain divalent ions, and content of substances with enhancing or inhibiting effects on the formation of HAs. In addition, there are other factors, which depend on the type of food, such as muscle tissue and the presence of certain genes (Alaejos *et al.,* 2011).

The relevance of cooking method to HAs formation relies on the food maximum internal temperature attained as this parameter is thought to combine the processes of heat transfer, water loss and cooking-surface reactions, which in turn affect HAs formation (Skog *et al.,* 1995a,b). Significant amounts of HAs begin to form in meats and model systems at temperatures of 150°C or higher (Bjeldanes *et al*., 1982; Knize *et al*., 1985; Skog *et al.,* 1997). Pan-frying, barbecuing and grilling/ broiling are high-temperature cooking methods, using radiative and conductive processes, with the greatest potential to generate significant amounts of these compounds. Oven-roasting and baking are methods that cook meat by indirect convection and at lower temperatures, thus producing lower or intermediate levels of HAs. Boiling, steaming, and stewing without previous browning or braising are cooking methods at temperatures at or below 100°C, that usually produce insignificant amounts of HAs (Bjeldanes *et al.,* 1983; Bogen *et al.*, 2001; Skog *et al*., 1998).

Data from the present study focused on PhIP, MeIQx and DiMeIQx as such compounds have been reported to be the most abundant (Augustsson *et al.*, 1997; Knize *et al.*, 1997; Skog *et al.*, 2002). The estimated HAs levels in meat and fish home cooked samples in Asia countries were generally lower than those reported for the Western diet (Jahurul *et al.*, 2010; Salmon *et al.*, 2006; Wong *et al.*, 2005). For example, concentrations reported for home-cooked samples in Spain (Busquets *et al.*, 2004) and Poland (Warzecha *et al.*, 2004) were 46.9 and 0.8 ng/g for PhIP and DiMeIQx in deep-fried chicken respectively, or 3.2 and 7.5 ng/g of for PhIP and DiMeIQx in pan-fried pork respectively. Due to differences in study design, intake frequency, cooking method, and cooking duration, it is difficult to make an accurate comparison of the daily intakes of HAs between this study and other reported data from other studies (Iwasaki *et al.*, 2010; Jahurul *et al.*, 2010; Salmon *et al.*, 2006). Nevertheless, in view of the preferred consumption of fried and grilled meat products and the variety of cooking methods for meat and fish, the estimated intakes of HAs in the present study were lower than those reported by other studies. Generally, the type and content of HAs in cooked poultry meat, red meat and fish vary with cooking method and cooking conditions (Cross *et al.*, 2004; Liao *et al.*, 2010; Sinha *et al.*, 1995). Thus, cooking practices in Cambodia may lead to lower

HAs levels in food, resulting in overall lower HAs exposure of the Cambodian population as compared to other developed countries.

Cambodian cooking is similar to other Asian countries, such as Chinese cooking methods which attempt to achieve "just doneness" to preserve the natural juices within the meat (Salmon *et al.*, 2006). Cooking at higher temperatures and for longer periods of time is well-known to increase the amount of HAs. Some studies reported that meat cooked below 150°C to rare or medium-rare doneness showed lower meat mutagen (such as HAs) contents than meat cooked over 150°C to well-done (Abdulkarim *et al.*, 1998; Murkovic *et al.*, 2004; Sinha *et al.*, 1998b). The individual HAs measured are not produced to the same extent by each cooking method and doneness level (Sinha *et al.*, 1998c). The internal part of the meat products showed lower HAs contents than the surface (Abdulkarim *et al.*, 1998; Dolara *et al.*, 1979). Their formation is highly dependent on various factors such as cooking temperature, cooking method, cooking time, type of meat, fat, and moisture content (Sugımura *et al.*, 2000; Skog *et al.*, 1995a,b), sugar, free amino acid and creatinine content of meat. In addition heat and mass transfer, lipid oxidation and antioxidants have consequences on HAs concentration (Pais *et al.*, 1999; Oz *et al.*, 2007). The formation of the crust is the result of steady transportation of water and dissolved compounds such as amino acids and creatinine to the surface by capillary flow to the evaporation zone; thus, the precursors of HAs are concentrated on or near the surface of the meat where there is the highest temperature. Prolonged cooking times at the same temperature induced a marked increase in the formation of some HAs (Gross *et al.*, 1992; Knize *et al.*, 2001; Miller *et al.,* 1983; Salmon *et al.*, 2000; Tran *et al.*, 2002). But MeIQx and PhIP showed an apparent decrease with time during pan-broiling (Gross *et al.*, 1992). Increases of degree of doneness caused increments in the content of PhIP and DiMeIQx, or of PhIP and MeIQx (Sinha *et al.*, 1995; Knize *et al.*, 2005).

Degree of doneness, which is often closely related to surface browning and total cooking time, is a key issue for production of HAs in cooked meat (Keating *et al.*, 2001; Sinha *et al.*, 1998c; Warzecha *et al.*, 2004). In numerous assessments of human exposure to HAs, photographs showing a variety of surface colours have been used to estimate the degree of doneness and indirectly the content of HAs (Sinha *et al.*, 2002). Colour development increases with cooking temperature, but no correlation existed with the content of HAs (Solyakov *et al.*, 2002); for instance, chicken breasts pan-fried at 190 and 220°C had similar colour measurements, but the amounts of MeIQx and especially PhIP differed markedly. Indeed, the relation between the degree of doneness and surface browning may differ because some people fry their meat at a high temperature for a short period of time to obtain a brown surface, while the interior is not cooked through. In the same way, it is possible to achieve a similar

degree of browning without applying excessive temperature and thus avoiding the formation of increased amounts of HAs (Kizil *et al.*, 2011).

Similarly to other Asian countries, marinating is a common practice in Cambodia,. The results from this study show that Cambodian cooking involves marinating cuts of meat with sugar, soy sauce, salt, pepper, garlic and oyster sauce (see **Chapter 3**). As other several Asian countries, Cambodian marinate is a traditional Cambodian cooking method that means immersing the food samples in a marinade for several hours, overnight or longer before cooking; thus, food is mixed with several kinds of ingredients, such as sugar, soy sauce, salt and seasonings (Lan *et al*., 2002; Lan *et al.*, 2004; Salmon *et al.*, 2006). Several studies have been reported about the spices, which are source of the natural antioxidants, could prevent the formation of HAs (Ogur *et al*., 1998; Balogh *et al*., 2000; Ahn *et al*., 2006). Virgin olive oil, cide vinegar, sugar, lemon, rust garlic, spice-marinades, red and black pepper, can be effective inhibition of HAs formation (Persson *et al*., 2003; Salmon *et al*., 1997; Smıth *et al*., 2008; Oz *et al*., 2011a, 2011b). Marinating prior to cooking is used frequently for pork, fish, chicken and beef, and usually goes before grilling or pan-frying, a relatively high-temperature cooking practice; these high temperatures have been associated with unusually high levels of PhIP (Sinha *et al.*, 1995). Based on the results from FFQ, Cambodian population consumed meat and fish more often marinated (19% for pork, 34% for fish, 7.7% for beef and 5.6% for chicken) than fresh (0.8% for pork, 25% for fish, 3.4% for beef and 3% for chicken). Other studies on the effect of marinade in Western-style cooking suggest that marinating meat before cooking substantially reduces PhIP formation, although levels of other HAs such as MeIQx may be higher in marinated meat (Knize *et al.*, 1997; Salmon *et al.*, 1997; Tikkanen *et al.*, 1996; Wong *et al.*, 2005). Because PhIP is the HAs present in the highest concentrations, this may have implications for modifying overall HAs exposure at the population level. Nevertheless, great variations without any clear correlation with mutagenicity are observed in the amounts of HAs between chicken samples treated with the same or different marinades (Busquets *et al.*, 2006; Tikkanen *et al.*, 1996). In addition, the variations observed in the amounts of HAs between equivalent products make it difficult to estimate their concentrations in foods.

The retention (or addition) of water through marinating might inhibit the formation of HAs in a given mass of meat. Salt, which was included in marinate was shown to increase water retention when mixed into ground beef before frying, thereby affecting HAs formation possibly by altering meat internal or surface temperatures, or by affecting the transport of HAs precursors within the meat (Persson *et al.*, 2003). This protective effect of water may also explain three observations regarding fish and HAs concentrations in another study (Salmon *et al.*, 2006). First, fish lost significantly less

weight due to cooking than either type of chicken, even though it was cooked for a similar length of time. Second, fish contained significantly lower amount of PhIP and DiMeIQx, as well as total HAs than non-marinated chicken; fish was also significantly lower in MeIQx and both isomers of DiMeIQx than marinated chicken. Third, the cooking surface temperature was significantly higher for fish than for either type of chicken, or one might have predicted that HAs concentration would be higher as a consequence; however, just the opposite was observed. This seemingly paradox was also seen for the low HAs concentrations reported in a survey of "fast-food" products, which are cooked at high temperatures, but for a relatively short cooking time (Knize *et al*., 1995), a similar phenomenon to that noted here in fish. High water content in fish could mitigate the effects of a higher surface temperature and inhibit HAs formation. Reported values of natural water content in raw fish, ranging from 67-77% in one study (Burger *et al*., 2005) are only slightly higher than that reported for raw chicken thigh, 66% (FSIS, 2000), but this difference, coupled with the barrier to water loss that the fish skin provided, or perhaps greater water holding capacity of the fish muscle tissue itself, might account for some of these observed differences between fish and chicken. In our study, fish contributed more to the HAs intake than the chicken, not because of the high concentration of HAs in fish but it is due to a very high consumption of fish (58%) in the studied population when compared with chicken (9%) consumption.

Meat is marinated for a variety of reasons, including improvement of flavour, tenderness and moistness of the cooked product. For all the food samples, marinated juice contains higher amounts of HAs than marinated food (Lan *et al*., 2002; Lan *et al.*, 2004), and the content of each HAs increased with increasing levels of soy sauce (0 to 20%) or sugar (0 to 5%) (Lan *et al*., 2002). Soy sauce plays a more important role for formation of HAs than rock candy (also called rock sugar). The addition of glucose during marinating promotes the formation of HAs. However, in shin et al., study shows that the addition of 1.5 g oligosaccharides (fructooligosaccharide, galactooligosaccharide, and isomaltooligosaccharide), and inulin to ground beef patties inhibited total HAs formation by 50, 47, 46, and 54%, respectively. They also reduced overall mutagenicity by 52, 51, 48, and 59%, respectively. These studies confirm that oligosaccharides and inulin have the potential to reduce HA formation in cooked beef patties (Shin *et al*., 2003). Buckwheat and clover honeys (with respectively a high and low antioxidant capacity) were also tested for marinades; 30% honey in the marinade formulation was more effective in inhibiting formation of HAs and overall mutagenicity, but marinades containing buckwheat honey were the most effective (Shin *et al.*, 2006b). In another study, beefsteaks marinated with teriyaki sauce or turmeric–garlic sauce in Hawaiian style had lower PhIP and lower MeIQx levels than un-marinated meat, and these levels diminished with time. On the contrary, marinating with Western commercial honey barbecue sauce caused initially a slight

increase in PhIP and MeIQx that were decreasing with time (Nerurkar *et al.*, 1999). Also, the marinated soy sauce, sugar and oyster sauce provided meat colour which confused the doneness level (Wong *et al.*, 2005).

Another explanation for the lower HAs daily intake assessed in this study, as compared to previous reported studies, is that the Cambodian population consumes more fish cooked under high temperature (in terms of grilling and pan-frying) than red meat and poultry; indeed, marinated fish cooked at high temperature (such as frying and grilling) contained less HAs than chicken (Salmon *et al.*, 2006).

Estimation of the potential exposure of HAs confirmed that dietary habit (fresh or marinated), preference cooking method (grill and pan-frying) and preference of doneness level (well-done) may contributed to this finding. It is very difficult to compare our results with published data due to the difference in diets between countries, difference between methodologies, data availability, targeted population, selected food items or groups. However, and in spite of the difficulties mention above, the Cambodian exposure assessment of HAs seem lower in Cambodia as compared to the other countries in the regions as well as the developed countries.

4.3. Limits and uncertainty of this study

Dietary exposure assessment to HAs and BaP at the individual level requires information on the dietary intake of various food items and corresponding food composition table. A validated method for assessing dietary intake has been developed for the specific objective of this study. A 24-hour recall and a FFQ developed in food consumption survey in Cambodia provided a suitable food consumption database for the exposure assessment to BaP and HAs. However, the validity and reliability of these newly-developed tools to assess usual BaP and HAs exposures remain to be determined. In a recent study, Kobayashi *et al.* found that HAs exposure estimated using FFQ data was moderately correlated with that measured in hair samples; this suggests that usual intake levels of HAs could be measured using FFQs in epidemiologic studies (Kobayashi *et al.*, 2007).

A major uncertainty in HAs exposure assessment is the designation of HAs concentrations in the cooked meats. The concentration data on HAs collected to date have been obtained from meats cooked by researchers under conditions considered representative of how these meats are prepared by the public. Actually, no data on HAs concentrations in meats actually cooked in residential settings are available, so the validity of using the data from controlled cooking studies to represent HAs levels in meals prepared by the public is unknown (Keating *et al.*, 2000).

A second uncertainty pertains to the use of data on levels of PhIP, MeIQx, DIMeIQx and BaP in meat and fish that were obtained from the published literature. When such data were not available for a specific combination (meat cut X cooking method X degree of doneness), they were imputed from the existing data. Relative to the robust food consumption and the consumer's preference data obtained from 24-hour recall and FFQ survey respectively, the data on levels of PhIP, MeIQx, DiMeIQx and BaP in meat and fish were more limited. Of the 100 foods classified (400 food items) based on meat/fish, cooking methods and degrees of doneness included in the current study, concentration data were not available for 160 food items (40%), most of which were fish, marinated beef, marinated pork and marinated poultry types; in particular, BaP data were absent for all food types. In addition, the quality of the data (and original studies) is unknown.. In assessing dietary exposure, values for the missing data for several food items were excluded from the data analysis and/or extrapolated based on the concentration data available for similar meat/fish types and degrees of doneness. More than that several existing data of some food items did not relevant to the real food items, for instant, the in our study, we used the existing contamination data of sea fish for the fresh water fish due to the unavailable of the contamination data in freshwater fish. Overall, the existing data gaps in the HAs levels in meat/fish and the extrapolation/surrogating from the available data as described in this report present significant uncertainty in the exposure estimates. Consequently, dietary exposure estimates for HAs and BaP based on the concentration data that were collected from the literatures may provide several uncertainties.

5. Conclusions

The survey of Cambodian dietary intakes and specific meat/fish intakes indicated that this population is exposed to BaP (and possibly other PAHs) and HAs in their daily diet at lower levels than in other Asia countries, and in Western countries as well. This might be explained by differences in meat consumption between Cambodia and Western countries, and also by differences in cooking method preferences, cooking practices and doneness levels. In addition, differences in consumption habits contribute to the observed results.

This investigation showed that cooked pork, beef, poultry and fish in Cambodia contain several HAs, and that their levels vary widely depending on the cooking conditions, cooking practices and type of meat (e.g. fresh or marinated meat, fat and water content, presence or absence of other ingredients, etc.). The results will be highly valuable for devising at-home cooking methods and practices designed to reduce the concentrations of HAs. This HAs exposure assessment, although limited to

three compounds, provides a realistic picture of the major contributors to the exposure. Statistically significant differences were seen between the Capital residents and the other regions, and between the younger adults (25-34) and the other age-range groups, so that it could be concluded that high consumption of several specific food items was the major source of the HAs exposure. It was observed that three major factors contributed to the variability in the assessment results, they were adult body weights (body weight of women was lower than men), consumption frequencies of the different food groups, and variances of the input data.

The most prevalent pathways of BaP exposure from the dietary patterns of the Cambodian population were from vegetables, rice, meat fish and fat and oil. Another key finding was that fried and grilled meat, chicken and fish are major dietary sources for the total intake of HAs among Cambodians. On the other hand, consumption of marinated meat and fish contributed to the high HAs intake not because of their high concentration but due to the high consumption of these products..

Although the results from our study did not suggest a relationship between diet, the presence of colorectal carcinogens and CRC in the healthy young Cambodian, it confirmed that the diet is not a vehicle for chemical contaminants in this population. On the other hand, dietary habits of Cambodian population seemed to provide several carcinogen inhibiting factors.

Because the dietary patterns in Cambodia are quite different from the Westerns countries where the majority of studies on CRC have been done, future studies on the estimated dietary intakes of BaP (as well as other PAHs) and HAs should be carried out in order to clarify the relationship between the intakes of BaP and HAs and the risk of CRC among Cambodian. These studies should also take into account potential confounders, especially genetic variations in the metabolic pathways of contaminants, as well as secondary plant products such as phenolic acids that are known to have an impact on the HAs metabolism and on the cancer risk.

Chapter 5. General discussion

As previously detailed in the literature review chapter, colorectal cancer is a worldwide problem, especially in developed countries such as European countries. From epidemiological and animal studies, there is increasing evidence for the potential role of diet in CRC. Indeed, the diet might be responsible for up to 70-90% of CRC cases (Doll *et al*., 1981; Willett, 1995). The risk of developing colorectal cancer, in particular, has been strongly linked to multiple dietary and other lifestyle factors. Epidemiological studies show that consumption of diets with a high percentage of red meat and processed meat, in contrast to white meat, is associated with an increased risk of colon cancer (Norat *et al.,* 2002). On the other hand, consumption of vegetables, especially green vegetables, is associated with a decreased risk of colon cancer (Koushik *et al*., 2007; Smith-Warner *et al.,* 2006). The mechanisms by which consumption of red meat and green leafy vegetables modulates colon cancer risk have been detailed in **Chapter 1**. Therefore we conducted this work to investigate the global impact of dietary pattern, including endogenous and exogenous food chemicals, on CRC in Cambodia.

In the first part of our study (**Chapter 2**), we focused on the identification and characterisation of CRC in Cambodia, a developing country in Southeast Asia. Epidemiologic patterns of CRC in this country have not been studied adequately. From 2005 to 2010, 356 CRC new cases have been reported; over this period, the incidence rates per 100 000 were 2.36 and 2.82 in men and women, respectively. Interestingly, CRC incidence is lower in Cambodia than in European and Western countries as well as in other Asian countries. In the Asia-Pacific region, particularly in the developed or Westernised nations such as Singapore, Hong Kong, South Korea, Taiwan and Japan, increasing incidences of CRC have been reported (Sung *et al*., 2005; Chen *et al*., 2002; Chong *et al*., 2009). As discussed in **Chapter 2**, our results reveal that Cambodia is still a country with low risk of CRC, particularly for older individuals.

Another striking point of our results lies in the rather high CRC proportion (30%) in young Cambodian population (<40 years), being similar to values seen in many Middle-Eastern countries (Ansari *et al*., 2006; Isbister *et al*., 1992; Soliman *et al.,*1997) but much higher than those reported in Western countries (Bulow *et al*., 1980; Mitry *et al*., 2001). The high proportions of young CRC cases seen in Cambodia, and probably in many neighbouring countries in Asia, could be due to the young age-structure of these countries and the relatively low rates of CRC in older individuals (Ansari *et al*., 2006). Other possible explanations lie in changes of dietary habits for the young adult population, environmental factors such as the exposure to widely used pesticides, as well as in the

absence of a diagnosis system in Cambodia. Besides, the Cambodian population does not have the knowledge about CRC, so that this cancer is difficult to diagnose. As a consequence, we observed that younger adults (less than 40-year old) were more likely than older adults to be diagnosed with late stages CRC (stage III or IV), these late stages being more difficult to treat. A similar trend was reported in a recent US study on 600 000 CRC: people in their 30s were about 20% more likely than other age groups to be diagnosed when their cancers were stage III or IV, with stage IV being the most severe grade of the disease (Goodman *et al.,* 2011). According to this study, other factors that increased the risk for having an advanced cancer at diagnosis were being African-American or lacking health insurance. In Cambodia, there is no insurance system, so that all the expenses for health care are processed by people with their own money. Even though cancer treatment in Cambodia has advanced remarkably since the opening of Marie Curie Oncology Center in Russian Friendship Hospital in 2003, its database does not cover all cancer incidences in the whole country due to the outflow of many cancer patients to other Asian countries. Thus, the limit of the nationwide cancer registry and epidemiology study in Cambodia may not provide a whole understanding of CRC in Cambodia; however, our study provides the first knowledge on the real situation related to CRC in this country.

One cannot exclude that CRC development at younger age in the Cambodian population as compared to Western countries may be due to difference in ethnic groups. A recent study showed, compared to the Malays, the Chinese population presents a higher incidence but developed CRC at a later age, (Chong *et al*., 2009). The higher incidence among Chinese and the lower incidence among Indians living in Southeast Asia mirror the rates in the countries of origin even though both racial groups migrated more than three generations ago. Racial differences in this disease were also reported by Griffin *et al.* who noted a higher proportion of CRC in young black men and women compared to their white counter parts (Griffin *et al.,* 1991). These observations on racial differences suggest that genetic factors might have an important aetiological role in CRC (Wang *et al*., 2004), even though other factors such as diet or lifestyle may be also involved. Typical genetic syndromes associated with CRC include familial adenomatosis polyposis and hereditary non-polyposis colon cancer (WHO, 2003); genetic and familial aetiologies account for less than 20% of CRC in the US (Alabaster *et al*., 1972). Many studies concluded that carcinomas are rare before the age of 40 years, except in individuals with genetic predisposition or predisposing conditions (Cronjé *et al*., 2009; Khuhaprema *et al*., 2008).

Most studies have investigated the effect of specific food items (red meat, processed meat, etc.), food groups (fish, cereals, vegetables, etc.) or nutrients (calcium, iron, vitamin A, etc.) on the

development of CRC risk (Bravi *et al*., 2010; Newby *et al.,* 2004; WCRF, 2007). However this approach is limited as foods and nutrients tend to be correlated. Therefore, several authors have investigated the effect of diet on CRC by considering dietary patterns built up by factor analysis (Slattery *et al*., 1999; Terry *et al*., 2001), a method which creates dietary patterns by aggregating related variables, thus making the interpretation of dietary exposure easier. So, in order to investigate the dietary impact on CRC for the Cambodian population, the second part of this work focused on dietary patterns based on food consumption surveys using relevant questionnaires as detailed in **Chapter 3**. Dietary patterns can be defined as combinations of dietary components (namely food groups, food items, and nutrients) intended to summarize the total diet or the key aspects of the diet for the population under study (Randi *et al.,* 2010). Many of the prominent hypotheses for effects of diet on cancer risk have been derived from examination of the associations between dietary patterns and cancer rates in different populations around the world. It was noted in the 1970s that developed Western countries have diets high in animal products, fat and sugar, with simultaneously high rates of cancers of colorectum, breast and prostate (Key *et al.,* 2004). In contrast, developing countries typically such as Cambodia have diets based on one or two starchy staple foods (rice), low intakes of animal products, fat and sugar, and low rates of these 'Western' cancers such as CRC (Key *et al*., 2004). Few authors have produced data on the patterns associated with risk of incidence (Mizoue *et al*., 2005; Rouillier *et al*., 2005) or recurrence (Cottet *et al*., 2005) of adenomatous polyps.

Based on epidemiological studies, high dietary intakes of meat, processed meat and fat lead to excessive consumption of calories, resulting in overweight or even obesity, and is strongly related to the high indices of colon cancer in Western countries and urbanized regions (WHO, 2010). For example, epidemiological studies, cohorts as well as case-control studies have established a strong association between obesity and CRC (Bergstrom *et al*., 2001; Engeland *et al*., 2005; Gerhardsson *et al*., 1990; Kim *et al*., 2007; Moghaddam *et al*., 2007; Pischon *et al*., 2008; Rapp *et al*., 2005; van Kruijsdijk *et al*., 2009). In our study, the proportion of obese people (with BMI $\geq$ 30 kg/m^2) was only 2.4%. Most of the Cambodian population (80.5%) lives in rural areas, where people spend most of their time in the fields, having a daily activity (NIS, 2008). In contrast, our studied population faced the problem of malnutrition, especially women: 12% of subjects had a BMI < 18.5 kg/m^2, and 82% of these were women. Yet, in the same time, the mean total energy intake for the studied adult population was close to the regional and WHO recommendations (**Chapter 3**).

The geographic distribution of CRC follows the division between Westernised versus developing countries, and incidence rates are increasing in countries adopting Western-style dietary habits (Vainio *et al*., 2003). Mortality from colon cancer has rapidly increased in the past few decades in

Japan, and the increase has generally been ascribed to the Westernisation of the diet, characterized by high intake of fat and meat (Kono *et al*., 2004). In France, the E3N study concluded that dietary patterns reflecting a Western way of life are associated with a higher risk of colorectal tumors (Kesse *et al*., 2006).

CRC risk factors such as consumption of alcohol, red meat and processed meat have been evidenced by epidemiological and experimental animal studies. Hence, a daily alcohol intake over 40 g may increase CRC risk by more than 40% compared to non-consumers (Bagnardi *et al*., 2001; Huxley *et al*., 2009; Longnecker *et al.,* 1990; Moskal *et al*., 2006; Cho *et al*., 2004). In our study, among the 941 persons surveyed, only 5.6% regularly consumed alcoholic beverages, with a mean daily alcohol intake of 26 g; most people drank occasionally.

Presently, most epidemiological evidence is available for a detrimental role of meat and meat products (especially red and processed meat) in the aetiology of CRC (Bingham *et al*., 2004; Cross *et al*., 2007; Giovannucci *et al*., 1994; Linseisen *et al*., 2002; Wei *et al*., 2004). It has been suggested that this increased risk is due to the greater production of bile acids, formation of carcinogenic agents and toxic effects inducing the proliferation of colonocytes (Kushi *et al*., 2002). In dose-response relationship, a 29% increase in CRC is expected for daily consumption of either 100 g of red meat or 25 g of processed meat (Corpet *et al.,* 2011; Larsson *et al.,* 2005, 2006; Norat *et al*., 2002; Sandhu *et al.,* 2001; WCRF, 2007). To reduce the risk of cancer, the World Cancer Research Fund (WCRF) recommended in 1997 that red meat intakes shoild remain below 80 g/day, of which very little should be processed (WCRF, 1997). However, ten years later, it lowered this limit to 71 g/day or 500 g/week, with further emphasis that intakes of processed meat should be avoided completely (WCRF, 2007). This recommendation is generally consistent with the current red meat consumption levels based on national survey data (Cook *et al*., 2009; EFSA, 2010). Results of dietary pattern analysis in **Chapter 3** show that the studied Cambodian population consumed total meat with a mean value of 75 g/day, being close to the international recommendation (WCRF, 2007); we did not report any processed meat consumption. By way of comparison, the individual national food consumption (INCA2) survey showed that mean red meat and processed meat intakes were 370 and 270 g/week respectively in France (Volatier *et al.,* 2006); distribution data showed that near a quarter of the French adult population (39% men and 13% women) eats more red meat than the recommended 500 g/week, and a quarter eats more than 50 g/day processed meat (we do not know how much these two populations overlap). So it appears that the Cambodian population consumes less red meat than France and other European countries.

From epidemiological and experimental studies, other dietary patterns such as fish, cereals, vegetables and fruits were considered to protect against CRC (Rao *et al.*, 2001; Takahashi *et al*, 1997). Our study reveals that almost all studied Cambodian population (97%) consumes fish with an average of 75 g/day, mainly freshwater fish. Based on our study and other reports, it appears that Cambodia has the higher fish consumption amount Asian countries and in the world (Ahmed *et al*., 1998). The average fish consumption in Cambodia is above the international recommendation which calls for cooked fish consumption of 150 g (or 2 servings)/week as part of a healthy pattern of eating (FAO/WHO, 2001, UK. 2004, US, 2005).

Fruits and vegetables, rich in potentially anti-carcinogenic constituents such as vitamin C, carotenoids, folate, dietary fibers, flavonoids, plant sterols, phenolic acids, and other phytochemicals, have been hypothesized to reduce CRC risk (Steinmetz *et al*., 1991). Animal and human feeding studies support the hypothesized protective effect of fruits and vegetables on cancer by providing plausible biological mechanisms including reduction of oxidative damage to lipids and DNA, induction of phase I and II enzymes, and stimulation of DNA repair and apoptosis (Dragsted *et al*., 1993 and 2006). Epidemiological data overwhelmingly support the apparent inverse association between consumption of vegetables and fruits and cancer risk, as demonstrated in one comprehensive review of more than 250 case–control and cohort studies. These studies, conducted in countries with diverse dietary practices, evaluated risks for various types of cancer using a number of different dietary assessment techniques (Block *et al*., 1992; Smith-Warner *et al*., 1999). Some studies suggest that the benefit of vegetables and fruits consumption on CRC is due to fibers (Hill *et al*., 1998; Lin *et al*., 2005; Terry *et al*., 2001; WCRF, 1997). The EPIC study concluded that in populations with low average intake of dietary fibers, an approximate doubling of total fiber intake from foods could reduce the risk of CRC by 40% (Bingham *et al*., 2003). For instance, based on 25 years of follow-up data for men in seven countries, an increase in dietary fiber intake of 10 g/day was associated with a 33% lower CRC mortality risk (Jansen *et al*., 1999). The latest systematic review and dose-response meta-analysis of prospective studies concluded that a high intake of dietary fibre (up to 10 g daily of total dietary fibre), in particular cereal fibre and whole grains, was associated with a reduced risk of colorectal cancer (Aune *et al*., 2011).

In the studied Cambodian population, “vegetables and products” was the second food item the most consumed (after rice): on average, nearly all the studied adult Cambodian population (98.5%) consumed 250 g/day of vegetables. So Cambodia seems to stay in the higher level of vegetable consumption as compared to other countries in Asia, with a per capita vegetable intake level mong

the highest in Asia (Popkin *et al*., 1998). In addition, half of the studied population (55.8%) consumed fruits, with a mean consumption of 145 g/day of fruits. Taking into account both food items, around 50% of studied Cambodian population consumed vegetables and fruits as recommended internationally (400 g - or five servings - of fruits and vegetables/day). The world-estimated levels of current fruits and vegetables intake vary considerably from less than 100 g/day in developed countries to about 450 g/day in Western Europe (Lock *et al*., 2005). However, we must underline that fruit intake remains inadequate for near half (44%) of the studied Cambodia population, especially in rural areas; the same finding was found in many low and middle-income countries (Hall *et al*., 2009; IARC, 2001).

Dietary macronutrients such as dietary fat and proteins are well known to be promoter factors in CRC development. In contrast, dietary fibers and carbohydrates could protect against CRC. In our study, Cambodians consumed proportionately more carbohydrates but less proteins and fat (carbohydrates 68.8%, proteins 13.7%, fat 17.5%) compared to Western countries populations (carbohydrates 45%, proteins 16%, and fat 39%) (Lin *et al*., 2005; Mirnalini *et al*., 2008; Russell *et al*., 1999). Epidemiological and experimental studies gave evidence that high fat diet and/or Western style diet, with fat quantity up to 20%, may increase CRC risk (Giovannucci *et al*., 1994; Hursting *et al*., 1990; Rao *et al*., 2001; Reddy *et al*., 2000; Reddy *et al*., 1986, Reddy et al., 1984). In countries with high CRC incidence, the diet fat content represents about 40% of the total calories; this contrasts with low CRC incidence regions where the fat content is near 15-20% of total calories (Nagata *et al*., 2001). It is thus possible that fatty processed meat, red meat and Western style diet increase CRC risk because they bring too much energy to the consumer. Also, the quantity and composition of specific fatty acids influence this risk (Nkondjock *et al*., 2003).

Evidence from epidemiologic, clinical, and experimental studies also suggests that relatively high circulating levels of insulin might be associated with increased CRC risk (Jenkins *et al*., 2002). In this regard, diet carbohydrates may be particularly relevant because they have postprandial glycemic effects and can therefore affect insulin levels (Holt *et al*., 1997; Jenkins *et al*., 2002); glycemic index (GI) is a measure of this response, used to rank carbohydrate-rich foods relative to either white bread or glucose (Jenkins *et al*., 1981). Glycemic load (GL) is the product of GI with the amount of carbohydrate (in g) in a serving of a food (Foster-Powell *et al*., 2002). Several prospective cohort studies did not support the hypothesis that diets high in GL, carbohydrates, or sugar increase CRC risk (Larsson *et al*., 2007). Moreover, no association was observed between GL, GI or carbohydrate intake and the risk of distal colorectal adenoma (Oh *et al*., 2004). In a multiethnic cohort study, GL

and carbohydrate intake appeared to protect women against CRC, perhaps because a major source of GL was white rice (Howarth *et al*., 2008).

In summary, with regards to macronutrients, our results support the fact that the Cambodian dietary pattern is reach in carbohydrates and low in fat intake (17.5%), being in agreement with the health international recommendations. Also, the Cambodia population does not seem to consume excess protein intake, the major sources of proteins in the Cambodian dietary pattern being rice, meat and fish. The total amount of energy intake for the Cambodian population seems to be close to the value of the regional and international recommendations. We did not found any excess in energy intake for the studied population (**Chapter 3**).

Commonly consumed foods, particularly vegetables and fruits, are sources of numerous micronutrients. Several of these, including beta-carotene (a vitamin A precursor), vitamin E, vitamin C and selenium (all having antioxidant potential) as well as calcium, vitamin D (in fish, eggs and fortified dairy products) and folate, have been the focus of extensive experimental and epidemiological research to determine their influence on cancer risk (Greenwald *et al*., 2001; Oh *et al*., 2005; Steinmetz *et al*., 1996). Common green, yellow/red and yellow/orange vegetables and fruits contain more than 40 carotenoids (e.g. beta-carotene, lycopene, lutein and xanthins) that can be metabolized by humans (Khachik *et al.,* 1986, 2006). Epidemiological studies have shown that consumption of traditional Mediterranean diets, with significant amounts of vegetables (especially tomatoes), fruits, olive oil, grains, beans and fish, decreases the risk of developing chronic diseases such as heart disease and cancer. In particular, lycopene, a major component in tomato, exhibited potential anticarcinogenic activity in many types of cancer such as CRC (Guttenplan *et al*., 2001). Based on experimental and epidemiological studies, micronutrients such as beta-carotene, vitamin C, vitamins B or calcium can inhibit the endogenous formation of colorectal carcinogens and the pro-carcinogenic effect of heme. Antioxidant vitamins were suggested to have a protective role against CRC via inhibition of epithelial cell proliferation (Kim *et al*., 2002; Schatzkin *et al*., 1996).

In our study, we found that, among micronutrient intake, only vitamin C reached the reference daily intake. Calcium, iron and vitamin A intakes were lower than RNI. Our results do agree with other reports on the current intake of iron, calcium and vitamin A, as well as vitamins B1 and B2 as discussed in **Chapter 3**. A striking point is the limited overall calcium intake, being only approximately 64% of the recommended dietary allowance for adults in both sex and all age ranges (Barba *et al*., 2008; FAO/WHO, 2003). Due to the protective effect of calcium on CRC and because the low intake of calcium was found to be related substantially to food preference, it seems important

to develop culturally appropriate strategies to promote calcium consumption in Cambodia,. The most prominent dietary deficiency found in our study was for iron, particularly in women: the average intake was 12.5 mg/day for adult population, with a RNI achievement lower in women (44% of RNI) as compared to men (72.7% of RNI). In such cases of anemia in women, a diet with iron rich foods along with iron supplements is often recommended (Ramakrishnan *et al*., 2008). Iron-rich foods are oysters, poultry liver and red meat (Kongkachuichai *et al*., 2002; Lomboardi-Boccia *et al*., 2002). However, absorption of iron from food is influenced by multiple factors, especially the form of iron consumed. Heme iron, found in animal sources, is considered to be nutritionally important as it is higher in bioavailability (>15%) than non-heme iron (<5%), found in vegetable sources (Kalpalathika *et al*., 1991). Besides, even though the high dietary fiber intake of our participants may protect against CRC, it may also interfere with the bioavailability of micronutrients such as iron, zinc and calcium.

In this work, results on the analyses of nutrient intake were supported by food item and group analyses. The most favorable diet appeared to be a combination of more rice, vegetables and fish, and possibly less red meat (such as beef), milk, other cereals and fruits.

Look at to the dietary exposure on BaP in studied population, the presents result shows that the average intake of Cambodian population was lowest as compare to the other countries in the region as well to the other western country (detail in **Chapter 4**). It is well known that PAHs were the environmental contamination and also it may be created during cooked food (such as meat fish) at high temperature. The total daily intake of BaP due to the consumption of various food items investigated was estimated to be higher intake from rice, meat and fat.

For the studied Cambodian population, the consumption pattern of animal-origin foods (meat, poultry and fish), including amount, frequency, and cooking methods, differs substantially from that in Western countries. In the latter such as Europe, where a higher CRC incidence is observed as compared to Cambodia, populations consume more red and processed meat using high temperature cooking practices (roasting and grilling/barbecuing) than populations in Asian developing countries such as Cambodia, Malaysia, Singapore as well as China (Koh *et al*., 2005). For example, in the US, pan-frying was the most common method for cooking chicken (56%) and fish (54%), whereas between 34% (for steak) and 63% (for hamburgers) of beef was cooked by broiling/grilling (Bogen *et al*., 2001).

In order to investigate the global impact of dietary intake on CRC risk, assessment of dietary exposure to colorectal carcinogens was also performed in this work. Three heterocyclic aromatic amines commonly found in diet were considered (PhIP, MeIQx and DiMeIQx) as well as BaP as detailed in **Chapter 4**. Based on analytical data in the literature, we created a food contaminant database for the assessment of exposure to colorectal carcinogens. Not surprisingly, most of the meat items for which HA concentrations have been reported in the literature are fried or grilled (Skog *et al*., 1998). Hence, pan-fried meats have been identified as the largest source of HAs in the US diet, with chicken being the largest source among the various meat types (Keating *et al*., 2001); in contrast, in Japan grilled fish is a major dietary source of HAs (providing more than 50% of HA intake) (Kobayashi *et al.,* 2002).

As previously reported for Asian countries (including Cambodia) (Lee *et al*., 2009), our results show that the frequency and absolute amount of meat consumed, as well as the use of high-temperature cooking methods (related to HA levels in cooked meat), are much lower in Cambodia than in Western countries. Consequently, human exposure to colorectal carcinogens (HAs and BaP) *via* food consumption in Cambodia is lower than in these countries, such as European countries as described in detail in **Chapter 4**.

The current evidence is consistently proving that red meat, HAs and BaP intake not affects risk of CRC for Cambodian population. Although there are no study reported on Cambodians' risk for adenomas and intake of red meat, current evidence should be taken into consideration for the next future investigation, as Cambodian diets may increasingly becoming more westernized, especially in the capital, where meat was more consume than the other regions.

General conclusion and perspective

Colorectal cancer is a worldwide problem, being of major importance in Western countries and having a lower input in the developing countries. As diet might be responsible for increasing up to 70%-90% of this cancer, decreasing CRC risk should be possible by changing diet. The aim of this work was to better understand the CRC modulation by dietary pattern. We focused on the characterization of CRC, dietary intake and colorectal carcinogen intake in the adult Cambodian population. Understanding the real situation of these contaminants, dietary habits, and lifestyle of the studied population will provide us with a clearer view of the principle food sources that may present a health risk or have a protective effect.

Our results show a low consumption of red meat, coupled with a common high consumption of fish, for the studied Cambodian population. The dietary patterns observed in this study showed that a high dietary fiber intake provided by vegetables is a common dietary habit among Cambodians, with rice being the main contributor to carbohydrates. Our data on low red meat and no processed meat consumption agree with the fact that dietary habits of the Cambodian population have not dramatically changed towards a more Western style diet. This unique dietary pattern has been inversely associated with CRC and is perhaps an important factor in the low population incidence of CRC in Cambodia. Based on the international recommendation, dietary meat intake of Cambodian population does not seem to increase CRC risk.

Since consumption of red meat cooked at high temperature is relatively infrequent among the studied Cambodian population, especially in rural areas, we believe that red meat does not represent a significant source of HAs. Marinating red meat before grilling and frying is a common practice in Cambodia. This cooking practice appears to generate low HA levels. Historically, herbs and spices have enjoyed a rich tradition of use for the flavour enhancement characteristics and for their medical properties. Fresh spices and herbs such as fresh ginger, fresh garlic, lemon, curcuma and fresh herbs are common basic ingredients in most traditional Cambodian cooking, widely used in the whole country. Perhaps there is a more convincing evidence for a positive association of CRC with a Western dietary pattern among non-Asian populations, because the pattern is more strongly correlated with other CRC risk factors, such as obesity and physical inactivity. Differences in meat cooking methods between Western and Cambodian populations may be another reason for the discrepancy.

With regards to CRC, our data do not confirm lifestyle as a risk factor for CRC development in Cambodia. On the contrary, dietary habits in the Cambodian population appear to be protective against CRC development; adaptation of Western-style diet occurred only in a minority of the study cohort, mainly in those living in the Capital city. Yet, future studies are needed to explore the associations between dietary evolution, dietary intake and chronic diseases among Cambodian population.

The Cambodian dietary pattern was characterized by high consumption of rice and many plant foods. Traditional Cambodian foods (fish, fermented fish, soybean products, and vegetables) were found to provide high levels of carbohydrates and fibers, and low contents of fat. A diet rich in various plant foods could potentially reduce CRC risk because of their many biologically active chemicals. The Cambodian diet consists primarily of mixed dishes that are generally high in refined carbohydrates (e.g., white rice and noodles), fish, and green leafy vegetables, and contain relatively small quantities of meats, such as pork, beef and chicken. Indicative of a population in transition from low to high socioeconomic status, we observed a wide distribution of food intake, with traditional foods (e.g., rice and fish) mostly in the rural areas, and non-traditional foods (e.g., red meat and dairy) especially in the capital.

Although the diet patterns do not imply the typical Western high CRC risk, the component of the diet may be a vehicle of other food contaminants. Rice was the major food consumption for Cambodian population, which contributed more than fifty percent of the total energy intake; this food might also bring contaminants such as mycotoxins. Fresh vegetables are protective against CRC because of their fiber and micronutrient contents, but they may also contain environmental pollutants and pesticides. Genotoxic or carcinogenic substances can be formed or activated in the colon. Yet, a lot of future research is needed to understand the specific interaction of the dietary pattern and CRC in Cambodia. Data from epidemiological, preclinical and clinical intervention studies have contributed tremendously to the extensive body of evidence linking diet with CRC. However, we have only begun to scratch the surface. Current knowledge of the underlying basis of diet-CRC, genetic-CRC and relationships is minuscule or none in Cambodia. It should be possible to study and quantify diet-CRC associations to understand causes and effects by investigating the events in molecular biology and genetics that are important to diet-related carcinogenesis. This approach should complement epidemiological and metabolic cancer research designed to search for preventive or adverse effects of particular dietary constituents and to take into account inter-individual differences in genetic susceptibility.

Information from various types of investigations will continue to have an important role in clarifying the complex relationship between diet and CRC. However, greater attention to linkages between diet and genetics will enhance the opportunities for developing effective intervention strategies for CRC prevention. The future for understanding diet-and-CRC linkages will be expanded by the ability of the biomedical research community to use newly available technological advances to conduct basic research studies in molecular biology and genetics. This expanded approach for diet, life style, gene and CRC research is not simple; it has many implications and certainly cannot be implemented overnight. It will take motivation, dedication, collaboration and education and training across disciplines, as well as a concerted effort by nutritional scientists, molecular biologists, geneticists and clinical cancer researchers to achieve this vision. Although implementing such a paradigm admittedly will be a tremendous challenge, it is anticipated that the results will be exceptional and will move the research field of diet and cancer into a vitally important position in the battle against cancer.

The follow up food consumption survey, in order to identify the dietary assessment trend in Cambodia, is also a significant tool to investigate the evolution of CRC. On the other hand, using national data on food chemical contaminants for assessment of the dietary exposure to colorectal carcinogens is a major tool in assessing the risk in a given country. So, it is important, for further investigations, that Cambodia could use its own food contaminant database for assessing dietary exposure to chemical contaminants and for other food contaminants as well.

Cambodia needs its own 'National Cancer Institute (NCI)' for the three main objectives. First, with its standard quality of treatment and care, this NCI will be the referral center for all cancer cases in Cambodia. This will make possible the second objective, which is nationwide cancer registry and epidemiology study, as well as its own research studies. Last but not least, NCI will contribute to producing more experts in cancer-related fields by becoming a major cancer-training center. This will in return expand Cambodian cancer network through the distribution of young generation specialists in various cancer peripheral centers in some major provinces in Cambodia.

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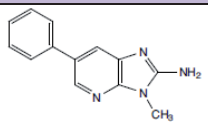
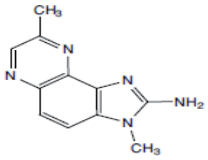
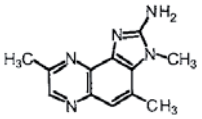
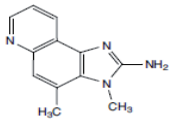
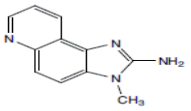
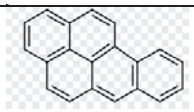
Annex

Annex 1

Annex 1.1. Chemical structure and physical properties of HAs and BaP

Name	Chemical structure	Molecular Formula and Weight	Water Solubility	Carcinogenicity
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine: PhIP		$C_{13}H_{12}N_4$ 224.26 g/mol	Insoluble	Group 2B
2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline: MeIQx		$C_{11}H_{11}N_5$ 213.2 g/mol	Soluble	Group 2B
2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline: DiMeIQx		C12H13N5 227.27 g/mol	Soluble	Group 2B
2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline: MeIQ		C12H12N4 212.2 g/mol	Slightly water soluble	Group 2B
2-Amino-3-methylimidazo[4,5-*f*]quinoline: IQ		$C_{11}H_{10}N_4$ 198.2	Insoluble	Group 2A
Benzo[*a*]pyrene[1]		$C_{20}H_{12}$ 252.3	Insoluble 0.0038 (mg/L; at 20C)	1

PhIP, MeIQx and MeIQ: is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals and supporting genotoxicity data.
IQ is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity from studies in experimental animals. Report on Carcinogens, Twelfth Edition (2011) (http://ntp.niehs.nih.gov/ntp/roc/twelfth/profiles/HeterocyclicAmines.pdf).

[1]: B[a]P is classified as a probable human carcinogen (Group B2) by USEPA (1994) and as probably carcinogenic to humans (Group 2A) by IARC (1987).

MeIQ, MeIQx, IQ, and PhIP are heterocyclic amines formed by condensation of creatinine with amino acids during the cooking of meat (Creatinine is a breakdown product of creatine, an important constituent of muscle.) All of these HAs share a common imidazole ring structure with an exocyclic amino group and, therefore, are known chemically as amino-imidazoazaarenes. Most HAs, including MeIQ, MeIQx, and IQ, are fully planar aromatic structures with no bulky out-of-plane functionalities; however, PhIP possesses a phenyl moiety that is not necessarily coplanar with the

main bicyclic imidazopyridine. All of these HAs occur as crystalline solids and are soluble in dimethylsulfoxide or methanol (US, 2002). MeIQ, MeIQx, IQ, and PhIP have no known commercial uses (IARC, 1993).

MeIQ, MeIQx, IQ, and PhIP are produced in small quantities for research purposes (IARC 1993). They are formed naturally during the cooking of muscle-derived foods (meat and fish) as by-products of the Maillard (or browning) reaction (Robbana-Barnat *et al.* 1996). It is postulated that the amino-imidazo part of HAs is formed from creatine, while the remaining parts of the compound are likely formed from Strecker degradation products, such as pyridines or pyrazines, which are formed in the Maillard reaction between hexose sugars and amino acids (Skog *et al.* 1998).

Benzo[*a*]pyrene, $C_{20}H_{12}$, is a five-ring polycyclic aromatic hydrocarbon whose metabolites are mutagenic and highly carcinogenic. BaP is listed as a Group 2B carcinogen by the IARC. It belongs to a class of polycyclic aromatic compounds known as benzopyrenes, which consist of a benzene ring fused to a pyrene molecule. BaP is a product of incomplete combustion at temperatures between 300 and 600 °C (572 and 1,112 °F) (Carrell *et al*., 1997). There is no commercial production or use of BaP. It occurs ubiquitously in products ofincomplete combustion and in fossil fuels.

As outlined in IARC (1983), BaP is metabolized initially by the microsomal cytochrome P-450 monooxygenase system to several arene oxides, which may rearrange spontaneously to phenols, undergo hydration to the corresponding transdihydrodiols, or react covalently with glutathione, either spontaneously or in a reaction catalyzed by glutathione-S-transferases. One of the phenolic metabolites, 6-hydroxybenzo[*a*]pyrene, is further oxidized to the 1,6-, 3,6-, or 6,12-quinones. The phenols, quinones, and dihydrodiols can be detoxified by conjugation to glucuronides and sulfate esters and the quinones can also form glutathione conjugates. In addition to conjugation, the dihydrodiols undergo further oxidative metabolism. Benzo[*a*]pyrene 7,8-dihydrodiol is in part oxidized to the 7,8-diol-9,10-epoxide, a compound considered to be the ultimate carcinogenic metabolite of benzo[*a*]pyrene (Faust, 1994).

Annex 1.2. Carcinogenicity in multiple organs of HAs in rat and mouse

Compounds	Species/Strain	Targets organs	References
PhIP	Rat, F344	Large intestine, Mammary gland, Prostate, Lymphoid tissue	Ito *et al*., 1991; Shirai *et al*., 1997
	Rat, SD	Mammary gland	Ghoshal *et al*., 1994
	Rai, CD	Mammary gland	El-Bayoumy *et al*., 1995
	Mouse,C57BL/6J	Large intestine	Nishikawa *et al*., 2005
	Mouse, Min/+pups	Large intestine	Andreassen *et al*., 2002
IQ	Rat, F344	Liver, small and large intestine, Zymbal gland, clitoral gland, skin	Takayama *et al*., 1984
	Rat, SD	Liver, mammary gland, Zymbal gland	Tanaka *et al*., 1985
	Mouse C57BL/6J	Large intestine	Nishikawa *et al*., 2005
MeIQ	Rat, F344	Large intestine, Zymbal gland, skin, oral cavity, mammary gland	Kato *et al*., 1989
	Mouse, C57BL/6	Liver, Large intestine	Fujita *et al*., 1999
MeIQx	Rat, F344	Liver, Zymbal gland, clitoral gland, skin	Kato *et al*., 1988; Kushida *et al*., 1994
		Large intestine	Kasahara *et al*., 1997
	Mouse, CDF1	Liver, lung, hematopoietic system	Ohgaki *et al*., 1987

Annex 1.3. Stage of CRC

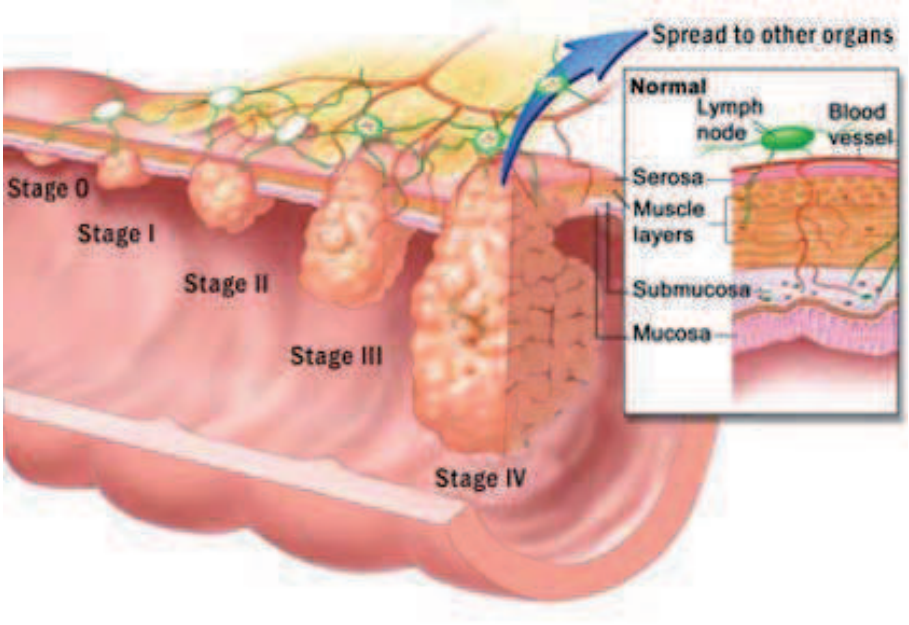


Figure 1.2. Difference stage of CRC (http://wegoodinfo.com/stage-4-colon-cancer/)

CRC offers 4 phases, and there are actually different treatments for just about every level. The best stage, called Stage 0, involves simply the lining belonging to the colon, which is generally known as mucosa. With this step, polyps, or even benign cancers, can possibly be removed 2 colonoscopy occurs. Once there're removed, the possibility of these people recurring won't exist.

In stage 0, abnormalcells are found in the mucosa (innermost layer) of the colon wall. These abnormal cells may become cancer and spread. Stage 0 is also called carcinoma in situ.

Stage I exists when the particular cancer arises past the particular lining towards walls on the colon or maybe rectum. The polyp that will never be treated will become a cancer, and actually reaches the wall belonging to the colon and also rectum. Treatments can include things like a medical operation to take away the cancerous a part of the intestines.

When that cancer actually gets to tissue that will surround this colon, but isn't going to reach the particular lymph nodes, the good news is Stage II large intestine cancer contained in the physique. When cancer malignancy spreads out of one section of the system to one more like thus, it is termed metastasis.

Subsequent, there is actually Stage III large intestine cancer. That's where the cancer malignancy spreads to arrive at the lymph nodes, however is not to some other organs inside the body. Treatment has to be more aggressive versus earlier stages of the cancer. The cancer has spread from the colon (with the lymph nodes) to distant organs and tissues such as the liver, lungs, peritoneum, or ovaries (Stage IV).

Annex 1.4. Foods with their insoluble, soluble, and total fiber content (g/ 100 g edible food portion).

Fiber Food Source	IDF	SDF	TDF
Cereals, nuts and seeds			
Rice	3.19	0.92	4.11
Wheat	9.64	2.84	12.48
Sorghum	8.03	1.64	9.67
Almonds	10.10	1.10	11.20
Sesame seed	5.89	1.90	7.79
Brazil nuts	4.10	1.30	5.40
Vegetables			
Bitter gourd	13.5	3.1	16.6
Field beans	9.3	2.1	11.4
Broad beans	7.3	0.8	8.3
Beet root	5.4	2.4	7.8
Cluster beans	6.1	0.6	6.7
Green plantain	5.8	0.2	6.0
Carrot	4.1	1.6	5.7
Cauliflower	3.5	0.7	4.2
Spinach	3.5	0.6	4.1
Potato	2.6	0.6	3.2
French beans	3.0	0.1	3.1
Onion	0.9	1.1	2.0
Fruits			
Guava	7.1	1.4	8.5
Jackfruit	2.1	1.4	3.5
Pomegranate	2.3	0.8	2.8
Pineapple	2.3	0.5	2.8
Apple, with skin	2.00	0.70	2.7
Grapes	1.3	1.3	2.6
Banana	1.80	0.60	2.4
Mango	1.06	0.74	1.8
Papaya	0.70	0.80	1.5
Pineapple, fresh	1.10	0.10	1.2
Pomegranate	0.49	0.11	0.60
Watermelon	0.30	0.20	0.50

From Khanum et al., 2000, Ramulu et al., 1997, Ramulu et al., 2003, Schakel et al., 2001.

Annexe 1.5

- The human microbiota.

The adult human gut is estimated to contain 100 trillions microbial organisms, collectively referred to as the microbiota (Savage, 1977; Suau *et al.,* 1999). The human microbiota is known to be dominated by strict anaerobes (Tiaskalova-Hogenova *et al*., 2004), with facultative anaerobes occurring in numbers approximately 1000-fold lower (Rastall, 2004). More than 500 different bacterial species may be present in the normal commensal microbiota, although the exact number and the variability among individuals remains an area of investigation (Hooper *et al.,* 2002). The colonic microflora has been suggested to have a critical role in setting the tone for a healthy bowel, including the risk for developing CRC (O'Keefe, 2008).

The human large intestine consists of the caecum, ascending colon, transverse colon, descending colon, sigmoid colon and rectum (**Fig. 1**).

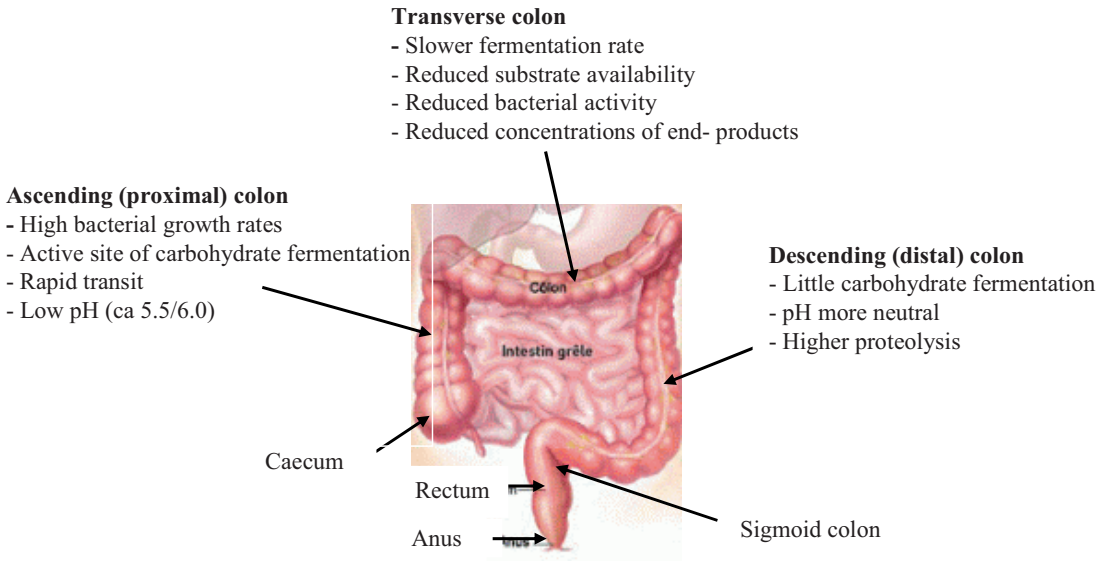


Figure 1.2. Regions of the human large intestine with corresponding bacterial activities and physiological differences (adapted from MacFarlane *et al*., 1991 and Vernazza *et al.,* 2006).

The pH varies all along the colon, with values in the caecum being approximately 5.7. This represents an important decrease compared to pH of 7-8 found in the ileum, and is attributed to the rapid bacterial fermentation of unabsorbed carbohydrates leading to the formation of short-chain fatty acids (SCFAs). The pH remains low in the ascending colon (5.6) and transverse colon (6.1) but then increases to 6.6 in the descending and sigmoid colon. The near neutral pH and the relatively low

absorptive state of this part of the colon further encourage extensive microbial colonization and growth (Vernaza *et al.,* 2006).

The total microbiota population outnumbers the cells in the human body and accounts for 35-50% of the volume of the colonic content or 41-57% of the dry weight of faeces (Salyer, 1979; Stephen *et al.,* 1980). Key physiological functions that might be related to cancer risk include control of epithelial cell proliferation and differentiation, production of essential nutrients and/or bioactive food components, prevention of overgrowth of pathogenic organisms, and stimulation of intestinal immunity (Tappenden *et al*., 2007).

- Signalling pathway of extracellular calcium.

The signaling pathway of extracellular calcium is illustrated in **Fig. 1**. Extracellular calcium acts as a genuine first messenger by binding the calcium-sensing receptor (CaR).

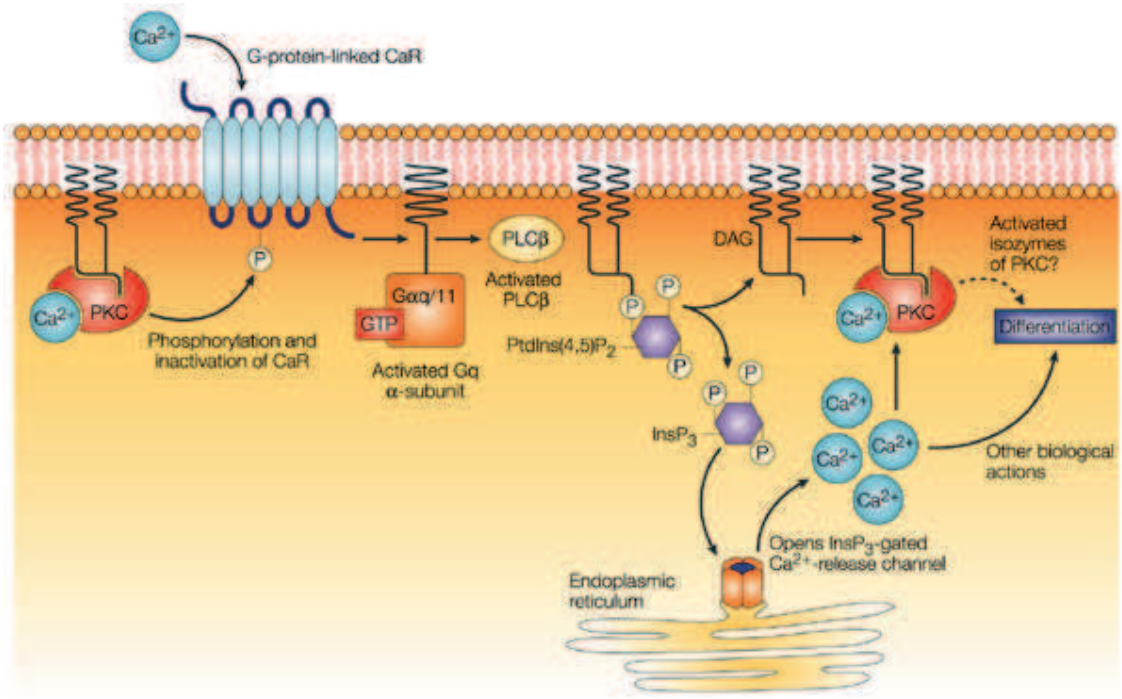


Figure 1.3. A signaling pathway of extracellular calcium.

CaR is a guanine nucleotide G-protein-coupled receptor consisting of the typical seven hydrophobic α-helices. Binding of calcium to CaR results in activation of phosphatidylinositol-specific phospholipase Cβ (PLCβ) through recruitment of the Gαq/11 protein. Activated PLCβ hydrolyses membrane-bound phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) to two important intracellular messengers, inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG).

$InsP_3$diffuses to the cytosol and releases calcium stored in the endoplasmic reticulum (ER) by binding and opening $InsP_3$-gated tetramer calcium channels embedded in the ER membrane.

An important effect of the signaling action of extracellular calcium is therefore a transient increase in the level of intracellular calcium, which results in a wide range of biological actions, conceivably including, together with DAG, the activation of isozymes of protein kinase C (PKC) that would promote the differentiation of intestinal cells. Although the proposed coupling of CaR with PKC activation is mechanistically based on valid premises, it remains to be proven experimentally. However, evidence is available that PKC can phosphorylate CaR on a threonine residue, and that this molecular event silences CaR, thereby effectively terminating the intracellular response to extracellular calcium challenge. As calcium can be a dangerous messenger, this might be a mechanism that cells use to control the duration and magnitude of the reactions described above (Lamprecht *et al*., 2003).

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Annex 2. Colorectal incidence rate in Cambodia

Age by Sex Distribution	Sex	CRC count	Population (census 2008)[1]	age specific rate =CRC count x 100 000/population
0-14-yrs	M	3	2314806	0.129600494
15-24 yrs	M	12	1503759	0.798000211
25-34 yrs	M	23	940752	2.444852629
35-44 yrs	M	30	752570	3.986340141
45-54 yrs	M	27	494916	5.455471231
55-64 yrs	M	29	279059	10.39206763
65-74 yrs	M	21	154459	13.59584097
75+ yrs	M	9	75733	11.884
Sub-Total	M	154	6516054	2.363393551
0-14-yrs	F	2	2198986	0.09
15-24 yrs	F	12	1484733	1
25-34 yrs	F	22	985844	2
35-44 yrs	F	39	829829	5
45-54 yrs	F	34	649460	5
55-64 yrs	F	59	389668	15
65-74 yrs	F	28	221325	13
75+ yrs	F	6	119783	5
Sub-Total	F	202	6879628	3
Total	M+F	356	13395682	3

[1]: *Institute National of statistic, ministry of planning, Cambodia*

Annex 3

Annex 3.1. 24 hours recall and food frequency questionnaire for food consumption survey in Cambodia

Name of interviewer …….	Name and sexof participant ……..
Date of interview ……..	Questionnaire number …….

I. Socioeconomic and demographic information and physical activity

1. What is your address and phone number?
……
……
2. What is your date of birth?
……
……
3. What is your current weight?
……
……
4. What is your current height?
……
……
5. What is your education level?
……
……
6. Currently, what is your profession? Specify (student, officer, farmer….)
……
……
7. Household size (how many people are there in your family?)
……
……..
8. Do you live near the factory? If yes, please specify type of factory
……..
……..
9. Are you a smoker (a daily or sometimes)? if yes, how many cigarettes are you usually smoking per day?
……..
……
10. In your family, are there any family members who smoke? a. yes (who?.....) b. no
……
……
11. How often do you drink alcoholic beverage (how many times per day, per week, per month or per year?) and how much each time? Specify the type of alcoholic beverage (beer, rice wine…)
……
……
12. How often do you use Aspirin?
a. regularly b. sometimes c. seldom d. never
……

……

13. Do you have or have had diabetic?

……

……

14. Do you have any family member who has or has had colorectal cancer? If you do, specify who.

a. yes (who?.....) b. no

……

……

15. Do you have previous history of colorectal polys/cancer? a. yes b. no

……

……

16. Do you have previous history of inflammatory bowel diseases? a. yes b. no

……

……

17. Do you use contraceptive hormone or drug? a. yes b. no

……

……

18. Physical activity: during one day or one week, how many hours do you spend

- Walking (including go to work, shopping…)? .....
- Running?.....
- Riding bicycle (including go to work, go to market…)? ……..
- Household task? ……..
- At garden, farm?
- At sport centre? ……
- And other sport (specify)?…….

Comment:

……..

……..

……..

……..

……..

……..

……

II. 24 hours recall questionnaires (Food consumption was recorded from wake up time till time goes to bed.)

Hours	Activity/ food consumption	Place	Food code	Consumption quantity		Comment
				Portion	Gram	
Morning						

Hours	Activity/ food consumption	Place	Food code	Consumption quantity		Comment
				Portion	Gram	
Noon and afternoon						

Hours	Activity/ food consumption	Place	Food code	Consumption quantity		Comment
				Portion	Gram	
Evening and night						

III. Foods frequency questionnaires (FFQs) for several specific food: pork, beef, poultry, fish, sausage and offal products

Food type	Cooking method	Code	Doneness level				How often					Consumption quantity		Comment
			R	M	WD	VWD	+1 T/D	1 T/W	2 T/W	3 T/W	1 T/M	Portion	Gram	
Bacon, fresh	G													
	PF													
	DF													
	B													
Bacon, marinade	G													
	PF													
	DF													
	B													
Pork, fresh	G													
	PF													
	DF													
	B													
Pork, marinade	G													
	PF													
	DF													
	B													
Beef, fresh	G													
	PF													
	DF													
	B													
Beef, marinade	G													
	PF													
	DF													
	B													
Chicken, fresh	G													
	PF													
	DF													
	B													

III. Foods frequency questionnaires (FFQs) for several specific food: pork, beef, poultry, fish, sausage and offal products, cont.

Food type	Cooking method	Code	Doneness level				How often					Consumption quantity		Comment
			R	M	WD	VWD	+1 T/D	1 T/W	2 T/W	3 T/W	1 T/M	Portion	Gram	
Chicken, marinade	G													
	PF													
	DF													
	B													
Duck, fresh	G													
	PF													
	DF													
	B													
Duck, marinade	G													
	PF													
	DF													
	B													
Other poultry (quail), fresh	G													
	PF													
	DF													
	B													
Other poultry (quail), marinade	G													
	PF													
	DF													
	B													
Fish, fresh	G													
	PF													
	DF													
	B													
Fish, marinade	G													
	PF													
	DF													
	B													

III. Foods frequency questionnaires (FFQs) for several specific food: pork, beef, poultry, fish, sausage and offal products, cont.

Food type	Cooking method	Code	Doneness level				How often					Consumption quantity		Comment
			R	M	WD	VWD	+1 T/D	1 T/W	2 T/W	3 T/W	1 T/M	Portion	Gram	
Fish, salted, dry	G													
	PF													
	DF													
	B													
Sausage, pork	G													
	PF													
	DF													
	B													
Sausage, beef	G													
	PF													
	DF													
	B													
Offal product, pork, fresh	G													
	PF													
	DF													
	B													
Offal product, pork, marinade	G													
	PF													
	DF													
	B													
Offal product, beef, fresh	G													
	PF													
	DF													
	B													
Offal product, beef, marinade	G													
	PF													
	DF													
	B													

III. Foods frequency questionnaires (FFQs) for several specific food: pork, beef, poultry, fish, sausage and offal products

Food type	Cooking method	Code	Doneness level				How often					Consumption quantity		Comment
			R	M	WD	VWD	+1 T/D	1 T/W	2 T/W	3 T/W	1 T/M	Portion	Gram	
Offal product, chicken, fresh	G													
	PF													
	DF													
	B													
Offal product, chicken, marinade	G													
	PF													
	DF													
	B													
Offal product, duck, fresh	G													
	PF													
	DF													
	B													
Offal product, duck, marinade,	G													
	PF													
	DF													
	B													

R: rare, M: medium, WD: well done, VWD: very well done
+1T/D: one time or more than one time per day
1T/S: one time per week
2T/S: two times per week
3T/W: three times per week
1T/M: one tome per month
G: grill, PF: Pan-frying, DF: deep-frying, B: bake

IV. Complementary questionnaires on fats, oil, sugar, condiments and species regularly use in Cambodia

1. How many kilo gram of salt does your family use per week?

……
……

2. How many kilo gram of sugar does your family use per week?

……
……

3. How many kilo gram of fats or oil does your family use per week?

……
……

4. How many kilo gram of mono sodium glutamate does your family use per week?

……
……

5. How many kilo gram (or litter) of say sauce does your family use per week?

……
……

6. How many kilo gram (or litter) of fish sauce does your family use per week?

……
……

7. How many kilo gram of oyster sauce does your family use per week?

……
……

8. How many kilo gram of garlic does your family use per week?

……
……

9. How many kilo gram of shallot does your family use per week?

……
……

10. How many kilo gram of pepper does your family use per week?

……
……

11. Other condiments, species and ingredients does your family use, kilo gram per week?

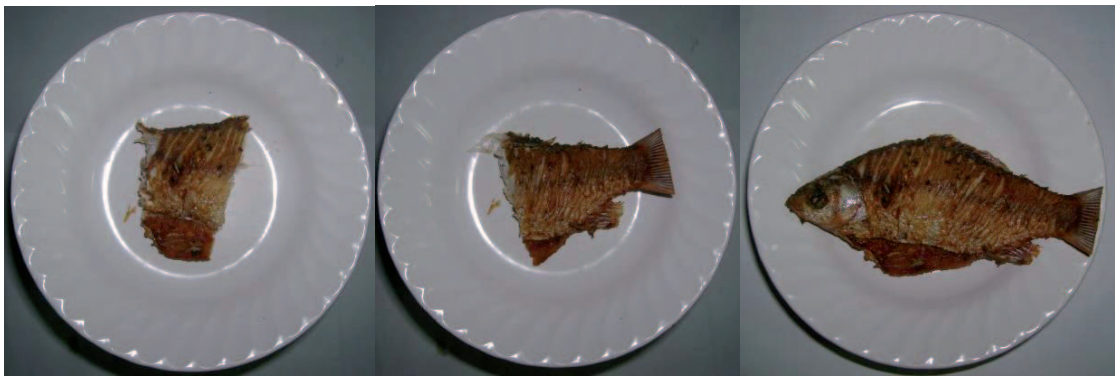
……
……
……
……
……
……
……
……
……
……

Annex 3.2. Food portion and photos for food consumption survey in Cambodia

(A) **(B)** **(C)**

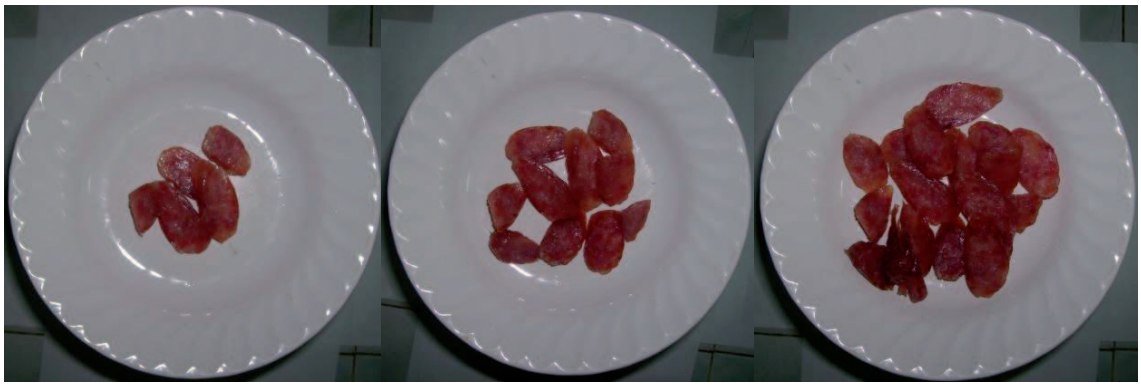
Annex 4.

(A) (B) (C)

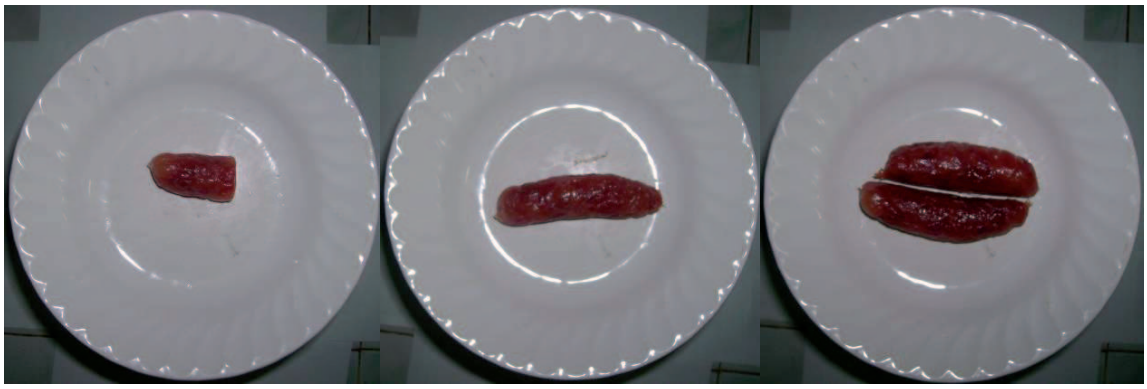


(A) (A) (C)

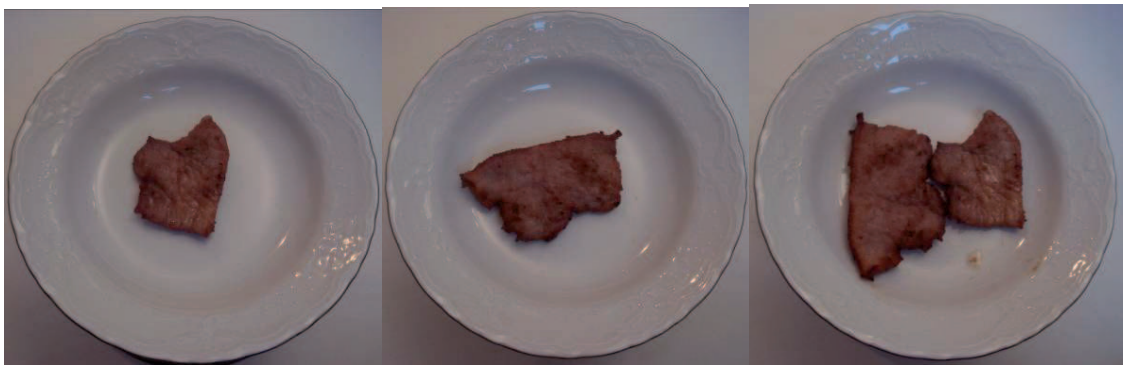
(A1) (B1) (C1)



(A) (B) (C)



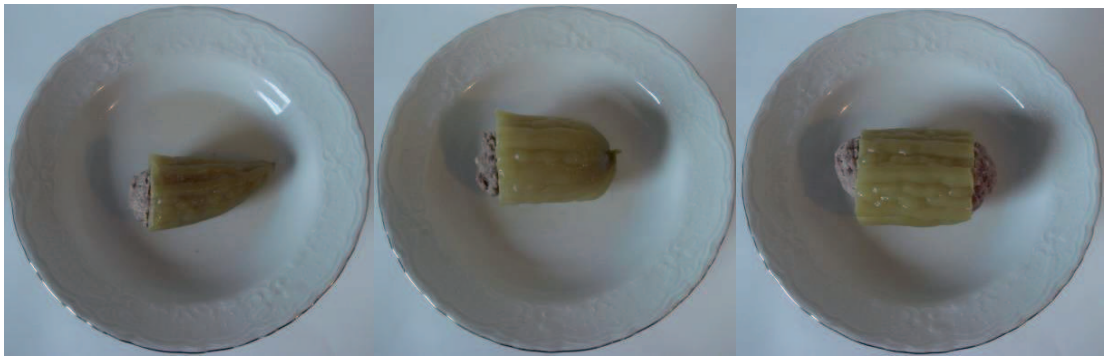
(A) (B) (C)



(A) (B) (C)

(A) (B) (C)

(A) **(B)** **(C)**



(A) (A) (A)

(A) (B) (C)

(A) (B) (C)

Population groups	Weight (kg)	Energy (kcal/day)	Protein (g/day)			Calcium (mg/day)	Iron (mg/day)		Zinc (mg/day)
			High Quality Protein Diet	Adjusted for 80% Protein Quality	Adjusted for 70% Protein Quality		7.5% Bioavailability	10% Bioavailability	Moderate Bioavailability
Infants (months)									
0-5	6	555	11	-	-	300 . 400	0.93	0.93	2.9
6-11	9	710	14	-	-	400	12.4	9.3	4.2
Children (years)									
1-3	14	1.180	16	20	23	500	7.7	5.8	4.8
4-6	20	1.470	21	26	29	600	8.4	6.3	5.7
7-9	27	1.825	27	34	39	700	11.9	8.9	6.0
Boys (years)									
10-12	34	2.110	34	42	48	1.000	19.5	14.6	6.8
13-14	47	2.650	45	56	64	1.000	19.5	14,6	8.9
15	47	2.650	45	56	64	1.000	25.1	18.8	8.9
16-18	56	2.980	49	62	71	1.000	25.1	18.8	8.6
Girls (years)									
10-12	36	2.010	35	44	50	1.000	43.6	32.7	6.1
13-14	45	2.205	41	51	58	1.000	43.6	32.7	7.2
15	45	2.205	41	51	58	1.000	41.3	31.0	7.2
16-18	49	2.240	40	50	57	1.000	41.3	31.0	6.8

Population groups	Weight (kg)	Energy (kcal/day)	Protein (g/day)			Calcium (mg/day)	Iron (mg/day)		Zinc (mg/day)
			High Quality Protein Diet	Adjusted for 80% Protein Quality	Adjusted for 70% Protein Quality		7.5% Bioavailability	10% Bioavailability	Moderate Bioavailability
Men (years)									
19-29	60	2.635	48	60	68	700	18.3	13.7	6.5
30-49	60	2.525	48	60	68	700	18.3	13.7	6.5
50-59	60	2.525	48	60	68	1.000	18.3	13.7	6.5
60-65	60	2.240	48	60	68	1.000	18.3	13.7	6.5
> 65	60	2.240	48	60	68	1.000	18.3	13.7	6.5
Women(years)									
19-29	50	2.115	40	50	57	700	39.2	29.4	4.4
30-49	50	2.065	40	50	57	700	39.2	29.4	4.4
50-59	50	2.065	40	50	57	1.000	15.1	11.3	4.4
60-65	50	1.720	40	50	57	1.000	15.1	11.3	4.4
> 65	50	1.720	40	50	57	1.000	15.1	11.3	4.4
Pregnancy									
1st trimester	-	-	+6	+7.5	+9	1.000	*	*	5.5
2nd trimester	-	+360	+6	+7.5	+9	1.000	*	*	7.0
3rd trimester	-	+475	+6	+7.5	+9	1.000	*	*	10.0

Source: *Barba C.V.C and Cabrera M.I.Z. 2008. Recommended Dietary Allowances harmonization in Southeast Asia. Asia Pac J Clin Nutr, 17, 405-408*

Population Groups	Weight (kg)	IODINE (µg/day)	SELENIUM (mg/day)	VITAMIN A (µg/day)	VITAMIN D (µg/day)	VITAMIN C (mg/day)	THIAMIN (mg/day)	RIBOFLAVIN (mg/day)	NIACIN (mg/day)	FOLATE (µg/day)
Infants										
(months)	6	90	6	375	5	25	0.2	0.3	2	80
0-5	9	90	10	400	5	35	0.3	0.4	4	80
6-11										
Children										
(years)	14	90	17	400	5	30	0.5	0.5	6	160
1-3	20	90	22	450	5	30	0.6	0.6	8	200
4-6	27	120	21	500	5	35	0.9	0.9	12	300
7-9										
Boys										
(years)	34	120	32	600	5	65	1.2	1.3	16	400
10-12	47	150	32	600	5	65	1.2	1.3	16	400
13-15	56	150	32	600	5	65	1.2	1.3	16	400
16-18										
Girls										
(years)	36	120	26	600	5	65	1.1	1.0	16	400
10-12	45	150	26	600	5	65	1.1	1.0	16	400
13-15	49	150	26	600	5	65	1.1	1.0	16	400
16-18										

Population Groups	Weight (kg)	IODINE (µg/day)	SELENIUM (mg/day)	VITAMIN A (µg/day)	VITAMIN D (µg/day)	VITAMIN C (mg/day)	THIAMIN (mg/day)	RIBOFLAVIN (mg/day)	NIACIN (mg/day)	FOLATE (µg/day)
Men (years)										
19-49	60	150	34	600	5	70	1.2	1.3	16	400
50-65	60	150	34	600	10	70	1.2	1.3	16	400
> 65	60	150	33	600	15	70	1.2	1.3	16	400
Women										
(years)	50	150	26	500	5	70	1.1	1.1	14	400
19-49	50	150	26	500	10	70	1.1	1.1	14	400
50-65	50	150	25	600	15	70	1.1	1.1	14	400
> 65										
Pregnancy										
1st		200	26	800	5	80	1.4	1.4	18	600
trimester		200	28	800	5	80	1.4	1.4	18	600
2nd		200	30	800	5	80	1.4	1.4	18	600
trimester										
3rd trimester										

Source: *Barba C.V.C and Cabrera M.I.Z. 2008. Recommended Dietary Allowances harmonization in Southeast Asia. Asia Pac J Clin Nutr, 17, 405-408.*