Risk factors for Gallbladder Cancer in India

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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CERTIFICATE

I certify that the thesis titled '**Risk factors for Gallbladder Cancer in India**' submitted for the degree of Doctor of Philosophy by Ms. Sharayu Mhatre is a record of the research carried out by her during the period September 2009 to July 2016 under my supervision. This work has not formed the basis for the award of any degree, diploma, associateship or fellowship at this or any other institute or university.

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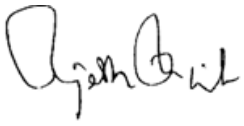

Dr. Rajesh Dikshit

Dedication

This dissertation is dedicated to my Late Beloved Mother Smt. Manda Mhatre and all those persons who lost their precious lives against cancer as well as to them who are battling against it with sheer determination and strong will power....

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'My research journey was inspired by my mother's battle with cancer,

which ignited me to pursue research in cancer'

This thesis has been completed with the support, encouragement & love of numerous precious people. It is my proud privilege and a matter of pride to express my gratitude to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me. I also express my sincere gratitude to all the study participants for their whole hearted co-operation and support as otherwise it would not have been possible to complete this study.

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Special gratitude from the bottom of my heart to the Almighty who helped & blessed me throughout this entire journey. I would like to pursue and continue my Epidemiological Research Work, which is always close to my heart. All this precious knowledge and experience that I have gained through hard work, will go a long way in not only building up my career but also in attaining the aims and objectives of my life in acquiring Ph D.

Thank you once again to one and all for making my Dream Come True!

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Synopsis

Homi Bhabha National Institute

Synopsis OF Ph. D. THESIS

Name of the Student: Sharayu Sitaram Mhatre

Name of the Constituent Institution: Centre for Cancer Epidemiology, Tata Memorial Centre

Enrolment No. : HLTH09200904003

Title of the Thesis: "Risk Factors for gallbladder cancer in India"

Board of Studies: Health Science

Introduction:

Gallbladder cancer is an aggressive malignancy with poor prognosis. The five year survival rate is less than 10% in most of the reported series [1]. Its incidence shows geographical variation throughout the world, with the highest being in South American countries, around the Mediterranean, Japan, Korea and Northern India while lowest being in Northern Europe, United States and Canada [2]. History of gallstone is an important risk factor for development of gallbladder cancer [3]. Obesity increases the risk for developing gallbladder cancer [4]. In addition, higher number of parity/gravidity has been related to increased risk of gallbladder cancer [5]. Gallbladder cancer has also been associated with infectious aetiology, particularly to chronic infection with *Salmonella & Helicobacter* species [6-10]. The role of genetic susceptibility in development of gallbladder cancer has been reported from few studies in India and China [11]. There is also some supportive evidence for the role of heavy metals in gallbladder carcinogenesis [12].

Gaps in literature:

The role of lifestyle and environmental factors in development of gallbladder cancer is not well documented, particularly in regions of high incidence. In addition, the role of chronic infection particularly with *helicobacter* species has been indicated but not studied in a properly designed large scale set up. The present study attempts to understand the role of lifestyle related factors and *Helicobactor pyroli(H. pylori)* infection in development of gallbladder cancer.

Hypothesis:

Life style factors are associated with increased risk of gallbladder cancer.

Aims and objectives:

1. **Primary:** To study the association of lifestyle related factors, gallstone and anthropometric measurement with gallbladder cancer.

2. **Secondary:** To study the association between infection with *Helicobacter pylori* and gallbladder cancer.

Primary aim:

To study the association of lifestyle related factors, gallstone, anthropometric measurement with gallbladder cancer.

Study population: Current study is a hospital based case-control study conducted at Tata Memorial Hospital (TMH), Mumbai from September 2010 to June 2015.

Selection criterion for enrolment of study participant: Cases were men or women patients with histopathologically and /or cytologically confirmed primary gallbladder cancer, aged 20-70 visiting TMH, Mumbai for diagnosis and/or treatment. The cases enrolled had a date of diagnosis not more than 12 months prior to the date of interview. Controls were all visitors with no history of cancer coming to different Disease Management Groups (DMGs) at TMH, Mumbai. The study was approved by the TMH Institutional Review Board (IRB). Written informed consent was obtained from all study participants before enrolment in the study.

Data Collection:

1. **Questionnaire based data Collection:** Study participants were interviewed by trained research staff using a pre-designed questionnaire. Information was obtained on demographic details, socioeconomic information, dietary intake, tobacco usage (smoke and smokeless), residential history, reproductive history and past medical history including history of gallstone. The data on all enrolled cases and controls were entered into the electronic database.
2. **Anthropometric measurements:** A height measurement was done using a 'drop down' tape measure fixed at about 2 metres on a wall using standardized protocol. Weight was measured using a calibrated instrument using standardized protocol. Waist circumference

(WC) was measured halfway between the costal edge and iliac crest and hip circumference (HC) was measured as the greatest circumference around the buttocks.

3. **Blood collection:** A 10 ml blood sample was collected from each study participant and fractionated into serum and red blood cells (RBCs). Serum sample was stored immediately at -80^{0}C and then transferred to -196°C in liquid nitrogen cylinder for long-term storage.

Quality Assessment:

1. **Preparation of instruction manual for questionnaire based data collection**: In order to ensure the quality of data collection by the research staff, an instruction manual was developed. The manual contains the comprehensive guidelines for data collection [13].
2. **Preparation of instruction manual for data entry in electronic database:** A comprehensive instruction manual was prepared to ensure consistency in data entry [14].
3. **Quality control monitoring for data collection and data entry:** All the questionnaires were regularly checked for completeness and consistency immediately after conducting the interviews and again after data were entered in an electronic database. A training program was conducted every quarter so as to ensure the quality of data collection as well as of data entry. Real time logical checks and double data entry were executed for the accuracy of data entry.
4. **Reproducibility of questionnaire:** Reproducibility of the main questionnaire was assessed with an abbreviated questionnaire. This abbreviated questionnaire assessed the information on constant variables (non-changing in recent time) such as number of pregnancies, height, vegetarian /non-vegetarian status etc. Reproducibility was assessed by re-interviewing 253 study participants. Education, tobacco usage (smoke and smokeless), and use of hormone releasing contraceptives showed 100% reproducibility.

Other important variable such as completed age, age at menarche, height, no of pregnancies showed more than 80% correlation in two measurements.

Exposure assessment:

- **High risk and low risk region:** In order to quantify the geographical variation in gallbladder cancer incidence, Indian states and territories were divided into high and low risk regions using incidence rates extracted from Population Based Cancer Registries (PBCRs) and Cancer Atlas of India.
    - High-risk regions: Bihar, Delhi, Himachal Pradesh, Punjab, Rajasthan, Uttarakhand, Uttar Pradesh, Assam, Tripura, Sikkim, Jharkhand, West Bengal
    - Low- risk regions : The remaining states and territories of India

Effect of length of residence in a high-risk region, was evaluated by stratifying study participants into the following mutually exclusive categories:

- Never lived in a high-risk regions (reference)
- Lived for a minimum of 1 year but less than 20 years in a high-risk regions
- Lived for 20 and more than 20 years but less than a lifetime in a high-risk regions
- Lifetime (If study participant has lived the entire course of his/her life in a high-risk regions)

- **Gallstone status:** Ascertainment of gallstone status was based on self reports of gallstone history by the study participant. Two different definitions were used for classifying study participant on gallstone status.
    - Self reported: as per reported by study participant i.e. either present/not present
    - Stringent gallstone definition: Gallstone history was ascertained using definition of self reported gallstone; however those gallstones diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to the date of

interview for control were categorized as “not present”. Otherwise was categorized as “present”.

- **Body mass index (BMI):** An obesity measure was calculated by dividing weight in kilograms by the square of height in meters. Effect of BMI on gallbladder cancer was evaluated using WHO categories on obesity for world population [15]. Accordingly, study participants were classified into following categories: <18.5, 18.5-24.9 (reference), 25.0-29.9 and ≥30.
- **Waist to hip ratio (WHR):** WHR was computed by taking the ratio of WC (in cm) and HC (in cm).Quartile cut–off points for the WHR were calculated within controls and utilized for analysis purpose.
- **Regular smokers/chewers**: A study participant who smoked/chewed for at least once in a week for at least six months in his/her life was defined as regular smoker/chewer for the study.
- **Tobacco *quid:*** The combination of tobacco, areca nut, slaked lime, and catechu was defined as “tobacco *quid”.*
- **Betel leaf *quid* with tobacco:** The combination of betel leaf, areca nut, slaked lime, and catechu with tobacco was defined as “Betel leaf *quid* with tobacco”.
- **Dietary intake:** Information on diet was assessed with the 77 –dietary items semi-quantitative food frequency questions, part of main questionnaire**.** The food frequency questionnaire accessed the consumption frequency of listed dietary items one year prior to the date of interview for controls and prior to diagnosis of gallbladder cancer for cases. For analysis, food items were grouped into classes and their weekly consumption was computed. Based on consumption among controls, intake was divided into quartiles and/or tertiles which were further used for analysis. For cooking medium, monthly

consumption was computed, which was then divided into tertiles based on consumption among controls.

Statistical Analysis:

Risk of developing gallbladder cancer was estimated by fitting unconditional logistic regression models. Risk of the following variables were studied by estimating crude as well as adjusted odds ratios(ORs) and their 95 % confidence interval: birth region, length of residence in high risk region, effect of migration from high to low risk region, anthropometric measurements, gallstone history, tobacco usage (smoke and smokeless), dietary intake and reproductive factors. Covariates were adjusted for following potential confounders such as age(continuous variable), current residential region (North, North-East, West, Central and South), education (Less than 5 years of schooling, ≥5 year of education), gender, smoke and smokeless tobacco usage(yes/no), gallstone history (present/not present), WHR (continuous variable), and number of full term pregnancies(continuous variable).

Results:

A total of 1,170 gallbladder cancer cases and 2,525 controls were included for the statistical analysis.

Residential history: The OR of developing gallbladder cancer for those reporting birth in a high risk region was observed to be 4.82 (95% CI, 3.87-5.99) compared to those born in low risk region, after adjusting for potential confounders. A dose response relationship, with increased risk with increased length of residence in a high-risk region was observed ($OR_{lifetime}$=5.58; 95% CI, 4.42-7.05). The risk persisted even if study participant migrated from high to low risk region (OR= 1.36; 95% CI, 1.02-1.82).

Gallstone: The OR associated with self-reported history of gallstone was 28.94(95% CI, 21.55-38.86) after adjusting for potential confounders. However, reduction in risk was

observed using a more stringent definition of gallstone (OR $_{Stringent\ definition\ of\ Gallstone}$ =6.10; 95% CI, 4.34-8.56).

Central obesity: A higher WHR (men ≥0.97; women ≥0.95) was associated with increased risk of gallbladder cancer in both genders (OR $_{Men}$=1.93; 95% CI, 1.30-2.86 and OR $_{Women}$=4.68; 95% CI, 3.20-6.86).

Tobacco usage: Increased risk of gallbladder cancer was observed with longer duration of bidi smoking ($OR_{>20\ years}$ =2.13; 95%CI, 1.24-3.65). In chewers, length of time tobacco *quid* was found to have strong effect on gallbladder cancer risk ($OR_{>19\ years}$ =1.43; 95%CI, 1.05-1.94). Similar pattern of increased risk were observed for Betel leaf *quid* with tobacco chewers.

Dietary factors: The highest quartile of fruit and vegetable consumption was observed to give protection from gallbladder cancer compared to consumption in the lowest quartile (OR_{fruits} = 0.53; 95%CI, 0.41-0.69 & $OR_{vegetables}$ = 0.63; 95%CI, 0.49-0.80). No increase in risk was observed for higher consumption of chillies and pickles. Fresh fish intake was observed to be associated with increased risk of gallbladder cancer (OR_{fish} = 1.41; 95%CI, 1.10-1.80). Consumption of mustard oil in the highest tertile was observed to increase gallbladder cancer risk compared to no consumption of mustard oil ($OR_{mustard\ oil}$ = 3.41; 95%CI, 2.73-4.25).

Reproductive factors: Number of full term pregnancies was positively associated with gallbladder cancer after adjusting for potential confounders. Compared with women who had one child, those having four and more children had 2.34-fold risk (95% CI, 1.46-3.74) of gallbladder cancer. Women who began menstruating after the age of 12 years had a 1.61-fold risk (95% CI, 1.09-2.36) of gallbladder cancer , compared with those with menarche age at 12 years or younger, with significant linear trend (P-*trend*=0.04).

Secondary aim

To study the association of *Helicobacter pylori* with gallbladder cancer risk.

Methodology: Commercially available Pyloriset EIA-G III was used to determine *H.pylori* IgG antibodies in the serum of study participants. The Pyloriset EIA-G assay determines *H.pylori* IgG antibody titer in serum by means of indirect enzyme linked immunosorbent assay (ELISA). Assay has 100% sensitivity and 94.3% specificity. ELISA was performed using automated liquid handling system integrated with micro-titer plate washer and reader. Absorbance was measured at 420 nm.

Quality Control measures: Stringent quality control measures were followed at various stages of the assays, such as laboratory operations, quality control of serum samples and assay validity criterion. Two quality control samples (positive and negative) were included in each plate to ensure accuracy of results. Serum samples were tested in duplicate. If the absorbance values of duplicates have a coefficient of variation (CV) more than 7 %, the concentrations obtained with the absorbance data for that dilution was excluded from the calculation of the antibody concentration of the sample.

Analysis: Point to point calibration line on a semi-logarithmic scale using the absorbance of the reference standards: the units of the reference standard on the x-axis (logarithmic) and the respective absorbance on the y-axis was generated using program software Magellan^{TM}. Best fit calibration line was obtained using linear regression analysis. The absorbance readings are proportional to the logarithm of the antibody concentration (U/ml). Mean absorbance reading of the reference standards and patient serum samples were calculated.

Interpretation of ELISA assay: If the U/ml or absorbance of the serum sample is equal to or higher than that of the reference standard 2 (i.e. 20 U/ml and/or approximately 0.300), the result was considered to be positive for *H.Pylori* IgG antibody or else negative.

Statistical analysis:

Unconditional logistic regression was used to assess association between gallbladder cancer and *H. pylori*. OR and their corresponding 95% CI were used to estimate relative risk after adjusting on potential confounders. In order to avoid, any arbitrariness in selection of cut-off points, study participants were classified into four groups of increasing antibody titters, calculated using quartiles of antibody concentration within controls.

Results:

A total of 859 gallbladder cancer cases and 905 controls were tested for *H.pylori* antibodies in serum samples. Final statistical analysis was performed on 833 gallbladder cancer cases and 818 controls after exclusion of 113 samples based on quality control measures. No statistical significant association was observed for infection with *Helicobacter pylori* and gallbladder cancer (OR $_{helicobacter\ pylori}$=1.07;95% CI, 0.81-1.43).

Summary and Conclusion:

1. The current study is the first to demonstrate the role of the following lifestyle related factors in development of gallbladder cancer
    - Birth place: Birth in a high risk region was observed to increase the risk of gallbladder cancer and risk persisted even after migration from high to low risk region.
    - Central obesity: Higher WHR was observed to increase risk of gallbladder cancer. Higher body size in early childhood and /or adulthood was associated with increased risk of gallbladder cancer.
    - Dietary intake: Higher consumption of fruits and vegetables was associated with reduced risk of gallbladder cancer. Whereas, use of mustard oil as cooking medium observed to increase risk of gallbladder cancer.
    - Tobacco usage: Different types of tobacco usage (smoke/smokeless) were associated with increase in risk for gallbladder cancer.

2. Study also confirms increase in risk with history of gallstone and higher number of pregnancies.
3. *Helicobacter pylori*: No statistical significant association was observed between serum level of *H. pylori* antibodies and gallbladder cancer.

The study provides strong evidence for prevention of gallbladder cancer by tobacco control, central obesity reduction, and moderate consumption of mustard oil and fresh fish.

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List of abbreviation

BMI	Body Mass Index
CI	Confidence Interval
GBC	Gallbladder cancer
HC	Hip Circumference
IARC	International Agency for Research on Cancer
NIH	National Institute of Health
OC	Oral Contraceptive
OR	Odds Ratio
SNP	Single Nucleotide Polymorphism
TMH	Tata Memorial Hospital
WC	Waist Circumference
WHO	World Health Organization
WHR	Waist-to-hip ratio

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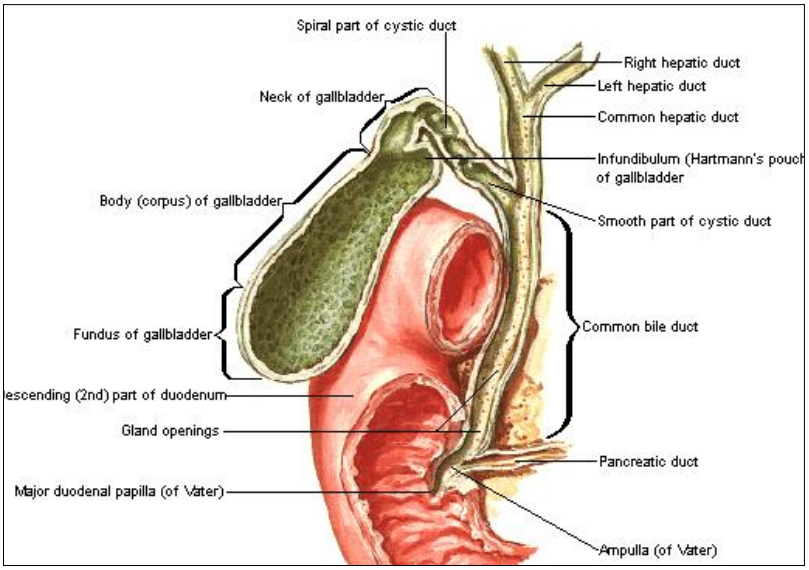
Chapter 1
Introduction

1.1 Biology of Gallbladder cancer

1.1.1 Anatomy of Gallbladder

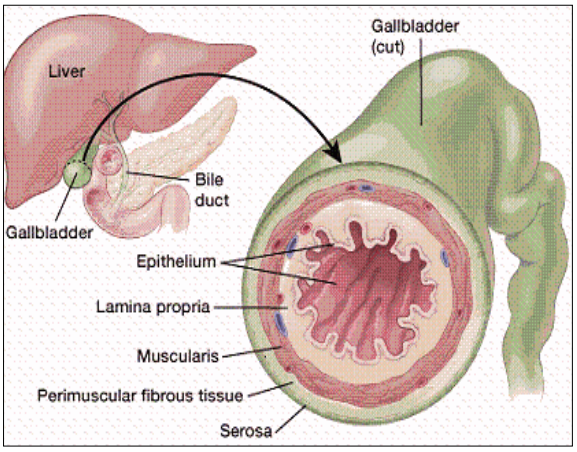
The gallbladder is a pear shaped organ situated in a fossa on the liver under surface. It is variable in shape and volume. Normally present at the junction of segments 4 and 5 (and at the lower limit of the principal plane or Cantlie's line) its position in relation to the liver may vary. The gallbladder is divided into a fundus, a body and a neck or infundibulum(1). The fundus is the blind, wide end of the gallbladder, the body makes up the majority of the organ, and the neck is the narrow, tapered end which is contiguous with the cystic duct and drains through the spiral valve. This hollow, muscular small organ is only about 3 inches in length and 1.5 inches in width at its widest point. The larger end of the gallbladder extends inferiorly and to the right while the tapered end points superiorly and medially. The tapered end of the gallbladder narrows into a small bile duct known as the cystic duct. The cystic duct connects to the common hepatic duct that carries bile from the liver. These ducts merge to form the common bile duct that extends to the wall of the duodenum(2). **[Figure 1.1]**

Figure 1.1 Location of Gallbladder



The histology of the gallbladder consists of mucosa with a single layer of epithelial cells, a lamina propria, a single layer of muscle that resembles the muscularis mucosa of the gastrointestinal tract, and a serosal layer(3) **[Figure 1.2]**.

Figure 1.2 Structure of Gallbladder



The mucosa, which forms the innermost layer of the gallbladder, lines the gallbladder with simple columnar epithelial tissue. The columnar epithelial tissue contains microvilli on its surface, increasing the surface area and allowing the lining to absorb water and concentrate the dilute bile.Beneath the columnar tissue is a thin lamina propria layer made of connective tissue and capillaries that support and anchor the epithelial layer. Deep to the lamina propria is the muscularis layer that contains smooth muscle tissue. Contraction of the muscularis pushes bile out of the gallbladder and into the cystic duct. Surrounding the muscularis is a thin layer of fibrous connective tissue that helps to reinforce and strengthen the wall of the gallbladder. Finally, the serosa forms the outermost layer of the gallbladder. The serosa is an epithelial layer that forms part of the peritoneum, or lining of the abdominal cavity. The serosa gives the gallbladder a smooth, slick surface to prevent friction between moving organs (4). Approximately 70% of the gallbladder carcinoma originates in the fundus of gallbladder, with 20% occurring in the body and 10% in the neck (5). Blood to the gallbladder is supplied principally by the cystic artery, which typically branches from the

right hepatic artery. The cystic artery also supplies blood to the cystic duct; at this point it breaks up into two or four minor branches (these are called Calot's arteries), which supply blood to the gallbladder's cervix and to part of the cystic duct. Then these arteries divide into the superficial branch, which crosses over the left face of the gallbladder and the deep branch, which goes in between the gallbladder and the gallbladder fossa(3).The veins of the gallbladder neck (cystic veins) communicate with veins along the cystic and bile ducts. They may drain through the portal vein to the liver, or after joining the veins of the hepatic ducts and upper bile duct. In addition, small cystic veins from the fundus and body of the gallbladder may pass directly into the liver. Lymphatic drainage of the gallbladder courses to the hepatic nodes through the cystic lymph nodes, which are typically located near the neck of the gallbladder(6).

1.1.2 Pathology and pathway of tumour development

Two types of metaplasia can be seen in the gallbladder mucosa: pseudopyloric and intestinal. Both of these are believed to result from long-lasting chronic inflammation in the gallbladder, mainly produced by gallstones. Intestinal metaplasia shows stronger relationship with gallbladder cancer (GBC) and steadily increases in frequency with age(7).

Two distinct epithelial lesions, dysplasia and adenoma, are currently recognized as premalignant stages of gallbladder carcinogenesis. Several histologic types of dysplasia and carcinoma in situ are recognised in gallbladder. These lesions are considered to be precursors of the corresponding invasive carcinomas. (7).

Only a small numbers of carcinomas (0.14% to 1.1%) appear to arise from pre-existing adenomas (8,9).

Observations from the literature strongly support the idea that gallbladder carcinogenesis occurs infrequently from pre-existing benign tumours. The evidence indicates that the

adenoma-carcinoma sequence is less important in gallbladder and involves molecular alteration different from those observed in the dysplasia-carcinoma pathway (7).

Incidental carcinoma is defined as a GBC discovered during cholecystectomy for benign diseases, with incidence varying between 0.14% to 6.1% depending on high risk or low risk area(10–12).

1.1.3 Microscopy

Carcinoma of the gallbladder appears as an infiltrating grey white mass. Some carcinomas may cause diffuse thickening and induration of the entire gallbladder wall. The gallbladder may be distended by the tumour, or collapsed due to obstruction of the neck or cystic duct. It can also assume an hourglass deformity when the tumour arises in the body and constricts the lateral walls. Papillary carcinomas are usually sessile and exhibit a polypoid or cauliflower-like appearance. Mucinous and signet ring cell carcinomas have a mucoid or gelatinous cut surface. Although, any type of gallbladder cancer may show necrosis, undifferentiated giant cell and small cell carcinomas are usually the most necrotic. Submucosal growth is an important feature of signet ring and small cell carcinomas(13).

1.1.4 Histopathology and types of tumours

GBCs are classified according to the depth of infiltration as early (mucosal or muscular layer infiltration) and advanced cancers (subserosal or serosal infiltration). Early cancers are usually better differentiated than advance one. The most frequent histological type is adenocarcinoma, accounting for >98% of GBC cases, among which two thirds are moderately or poorly differentiated. The tubular and micropapillary subtypes are the most frequent(7). It has been reported that micropapillary carcinoma is rarer in western countries than in Japan and is characterised by longer survival after surgery resection(14,15). Other less frequent histopathological sites have been described are squamous, adenosquamous, carcinoid, small cell carcinoma, and gastrointestinal stromal tumours(13).

1.1.5 Types of tumours

a. Adenocarcinoma: The adenocarcinomas are sub divided into three grades: 1. well differentiated, 2. moderately differentiated, and 3. poorly differentiated on the basis of their differentiation with outcome. Histopathological variants of adenocarcinoma are papillary, mucinous, intestinal type, clear cell, signet cell, and adenosqamous carcinoma.

Histological variants of adenocarcinoma:

- Papillary adenocarcinoma: This malignant tumour is composed predominantly of papillary structures lined by cuboidal or columnar epithelial cells often containing variable amounts of mucin(13).
- Mucinous adenocarcinoma (MC): constitute 2.5% of gallbladder carcinomas. MC's are typically large and advanced tumours at the time of diagnosis and thus exhibit more-aggressive behaviour than ordinary gallbladder carcinomas. Immunophenotypically, they differ from conventional gallbladder adenocarcinomas by MUC2(Mucin 2) positivity and from intestinal carcinomas by an often inverse CK7/20 profile(16).The abundant mucin makes the tumour appear hypocellular.
- Cystadenocarcinoma: Cystadenocarcinoma of gallbladder is a rare disease with a few reports in literature. Cystadenocarcinoma refers to a unilocular or multilocular glandular tumour that may be the result of malignant transformation of a cystadenoma(17).
- Clear cell adenocarcinoma: This rare malignant tumour is composed predominantly of glycogen-rich clear cells having well-defined cytoplasmic borders and hyperchromatic nuclei(13).
- Signet-ring cell carcinoma: Cells containing intracytoplasmic mucin displacing the nuclei toward the periphery predominate in this variant of adenocarcinoma. A

variable amount of extracellular mucin is usually present. Lateral spread through the lamina propria is a common feature(13).

- Adenosqamous carcinoma: This tumour consists of two malignant components, one glandular and the other squamous. The extent of differentiation of the two components varies, but in general they tend to be moderately differentiated(13).

b. Squamous cell carcinoma: This malignant epithelial tumour is composed entirely of squamous cells. The extent of differentiation varies considerably(13)

c. Small cell carcinoma: this lesion is very rare and represent 0.2% of all nuroendocrine tumours (18).

d. Undifferentiated carcinoma: Characteristically, glandular structure are absent in this type of carcinoma. They are characterised by four histological variants. Those are spindle giant cell, nodular, small cell and osteoclast type.

e. Carcinosarcoma: This malignant tumour consists of a mixture of two components: carcinomatous and sarcomatous.

1.1.6 Routes of spread: loco-regional, and distant

Vascular, lymphatic, intraperitoneal, neural, and intraductal are leading routes of spread. Intraductal spread has better prognosis than others(19). Loco regional spread is more common than distant metastasis. Metastases usually occur in the liver, lymph nodes, adjacent organs and peritoneum. Lymph nodes are usually found in about 60% cases while metastasis in liver is about 76%-86% cases. Intraperitoneal spread is common with ascites, omental nodules and peritoneal implants. Lymphatic drainage from the gallbladder occurs in a predictive fashion and correlates with the pattern of lymph node metastasis seen in GBC. Initially, cystic duct and pericholedochal nodes are involved, followed by more distant metastasis to posterior nodes to the head of the pancreas and then to intraortocaval lymph nodes. This primary route is called the cholecystoretropancreatic pathway. A secondary route

of lymphatic drainage includes the retroportal and right celiac lymph node through the gastrohepatic ligament, called cholecysto-celic pathway. The third one is called cholecysto-mesenteric route consisting of a pathway from the posterior of gallbladder to the aortocaval lymph node via pancreas(20).

1.1.7 Molecular genetics alteration

Little insight is known regarding genetic and molecular level alterations in GBC. Earlier studies have suggested the participation of oncogenes, tumour suppressor genes, DNA repair genes and microsatelite instability and epigenetic alterations (represented mainly by methylation of promoter region of gene) in progression of GBC. However, prognosis of GBC at a molecular level is still ambiguous. Mutation of TP53 is found in the vast majority of invasive gallbladder carcinoma and is relatively common in later stage of disease(21–23). The expression of cyclin dependent kinase inhibitor p21 has been observed in 28% of GBCs and by itself, has no impact on patient survival. In the presence of P53 mutations, patients with no p21 expression survive longer than those with p21 expression. On the other hand, patients without p21 expression but with expression of p27 have a better survival rate than those positive for both p21 and p27(24). Tumour suppressor gene p16 inactivation is frequent in GBCs and also associated with poor prognosis of disease(25).Cyclooxygenase-2(COX-2) is involved in early events of gallbladder carcinogenesis. Increased vascularisation induced by COX-2 might be also responsible for poor prognosis (26). Microsatellite instability (MSI) is present from early stages of carcinogenic process and this is limited to a subgroup of patients(27–30). Another significant factor that plays important role in GBC as well as cholecystitis with adenomyoma is inducible nitric oxide synthase (iNOS). It plays role in induction, particularly in chronically inflamed tissue and can lead to gene mutation(31). The oncogene c-erb-B2 is believed to be associated neoplastic progression of GBC and its expression may be a marker for worse prognosis.

1.2 Descriptive Epidemiology

GBC, though generally considered rare, is the fifth most common malignancy of the biliary tract, accounting for 80% to 90% of biliary tract cancers (32). GBC shows marked ethnic as well as geographical variation in its incidence.

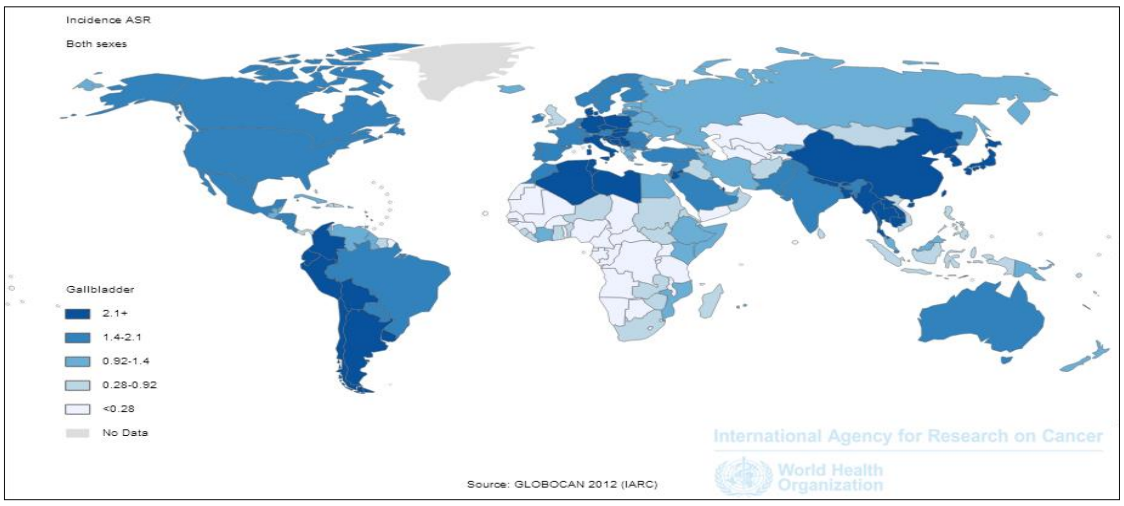
1.2.1 Burden of Disease

Worldwide 178,101 cases were diagnosed with GBC. The burden of GBC is higher in less developed regions with 116,000 cases than more developed regions with cases 63, 000 estimated by Globocan 2012. India itself has burden of 18,787 GBC cases which is 10% of its worldwide(33).

1.2.2 Incidence

GBC incidence is characterized by worldwide variation **[Figure 1.3],** being low in many European countries and U.S.A, relatively high in central European countries, and very high in Eastern Asia and South America.

Figure 1.3 Age standardized (world) incidence rate (per 100,000) of GBC (all ages, both sexes)



According to the incidence reported by CI5 monograph Chile was found to have the highest incidence of GBC among men and women: 11.3 and 25.1 per 100,000 persons respectively. Among the top 10 locations around the world, men seem to have very little geographic

variation, with the region of Korea and Japan making up of most of this **[Figure 1. 4]**. Geographic location for women however, shows more variation for GBC, with regions in Chile, Korea, Japan, China, U.S.A, Italy & India, among top 10 locations **[Figure 1.5].** Most of the 10 top locations for gallbladder cancer have an incidence of approximately 3.0 to 6.0 per 100,000 for both genders (34).

Figure 1.4 World standardized Incidence rate for GBC for men (top 50 locations)

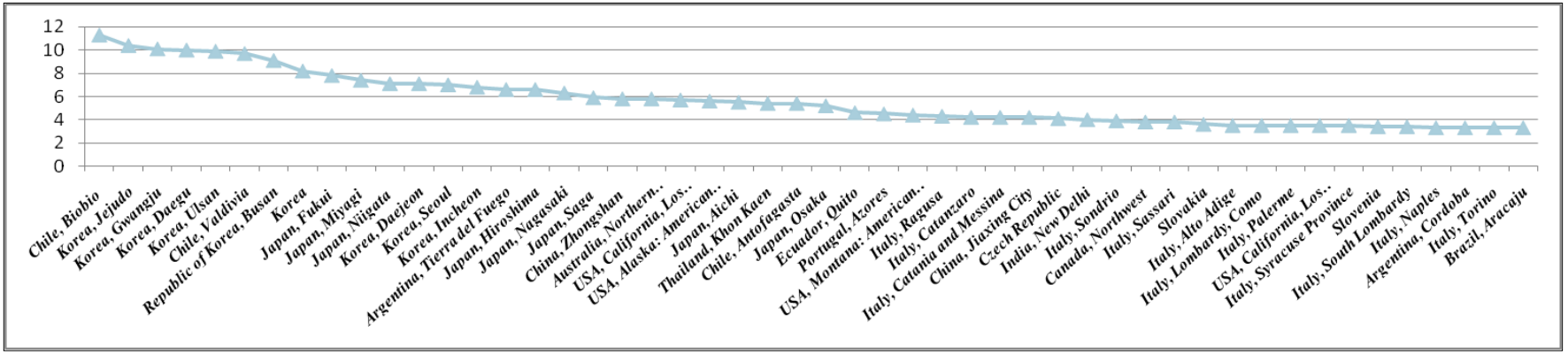
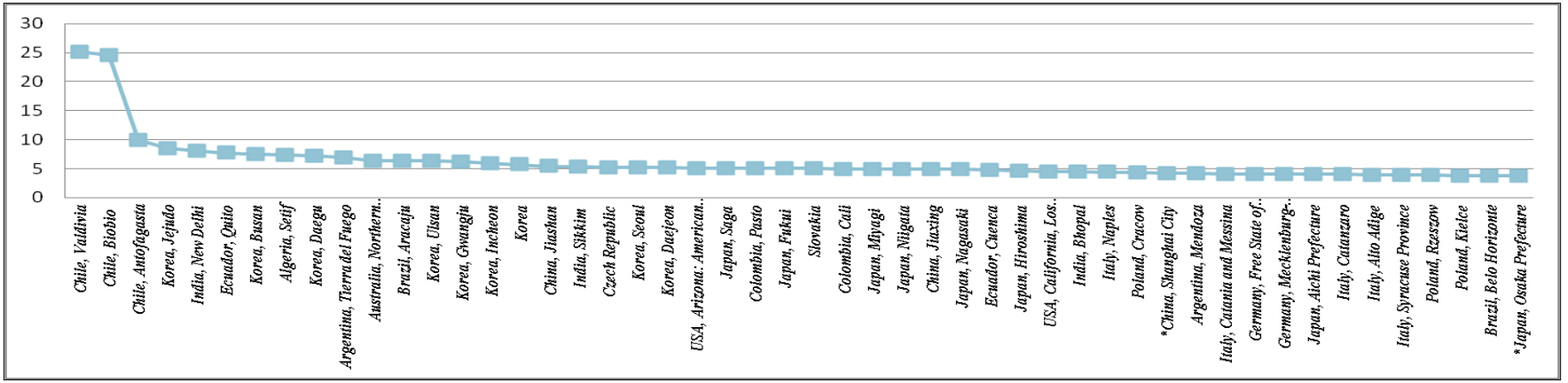


Figure 1.5 World standardized Incidence rate for GBC for women (top 50 locations)



Among women, incidence rates of GBC, among the top 10 locations around the world are higher than those for men .Highest incidence rates among women are twice than those of men, but incidence rate decline sharply for women than men **[Figure 1.4 & 1.5].**The Female/Male ratio of GBC incidence rates varies greatly: it is exceeded 5 in several high risk areas (e.g. India, Pakistan, Columbia and Spain) but was as well as in low risk areas (e.g. Denmark), but was typically between 2 and 3. F/M ratio was close to unity in Korea, Japan and some parts of China(34).

1.2.3 Survival

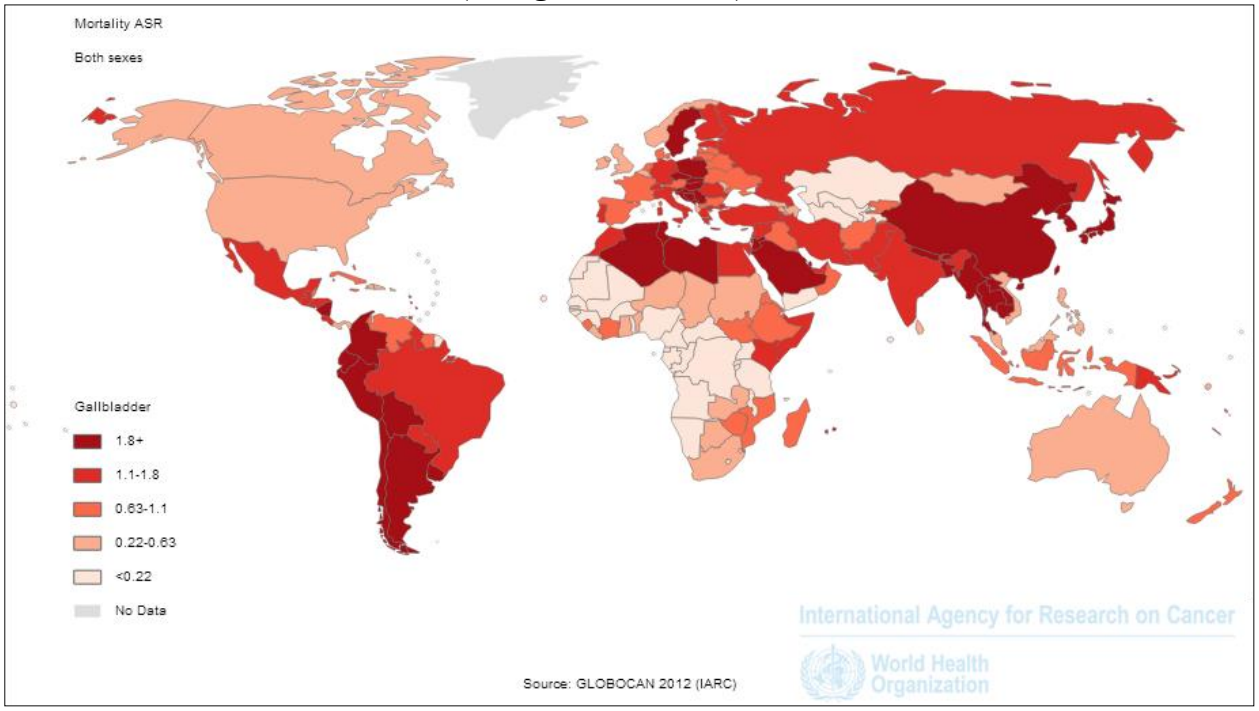
In a broad review of all published cases of GBC in 1978, a cumulative median survival of 5-8 months and 5-year survival rate of only 4% was reported. Over the past 3 decades, improved understanding of the characteristic of GBC, along with the advent of treatment modalities have resulted in better outcomes. Increased surgical experience and safe standardized technique of hepatic resection have resulted in a progressive decline in mortality and morbidity associated with this procedure(35). The American Joint Committee on Cancer "Cancer staging of manual" assessed 10,000 GBC patients diagnosed from 1989 to 1996, and reported improved 5 year survival rates of GBC. The five year survival rates are 80% for stage 0, then progressively fall to 50% for stage I, 28% for stage II, 8% for stage IIIa, 7% for stage IIIb, 4 % for stage IVa , and finally 2 % for stage iVb(32). Another study from Chile reported a survival rate of 92% at 5 years for early stage cancer patients. Whereas 5-year survival rate for those with advance cancer was only 24%. No differences were observed in survival with respect to sex, ethnic origin, or histological type(36).

1.2.4 Mortality

GBC mortality rates closely follow incidence: those with the highest incidence reflect the highest mortality **[Figure 1.6].** Worldwide burden peaks in the Chile (ASR: 7.8), closely followed by Bolivia (ASR: 7.5), Korea (ASR: 4.8) and Laos (ASR: 4.7)(33). This finding

is believed to be caused partially by the lack of access to health care in certain geographical locations. Mortality is declining some counties, like the U.S.A, Canada, Australia, and some parts of Europe (the UK and Hungary), but increasing in others, including Chile and Japan (32).

Figure 1.6 Age Standardized (world) Mortality Rate (per 100,000) of GBC (All ages, Both sexes).



1.2.5 Time trends in Incidence

Examinations of the time trend indicate a decline in GBC incidence rates over the last three decades. In the Scandinavian population, age-period-cohort-gender analysis indicated that the incidence rates decreased after the period of 1978-2012.Other European countries such as United Kingdom , France ,Germany ,and Czechoslovakia observed decline in GBC incidence rates over the period of last 3 decades.

Data from the United States show decrease in incidence over time for 1992-2007,with the Hispanic population showing gradual decrease in incidence(34).Incidence rates were even declined among a highly prevent GBC population, American Indian natives in New Mexico, United States over the period of 1981-2008(37). However, a slight elevation in incidence

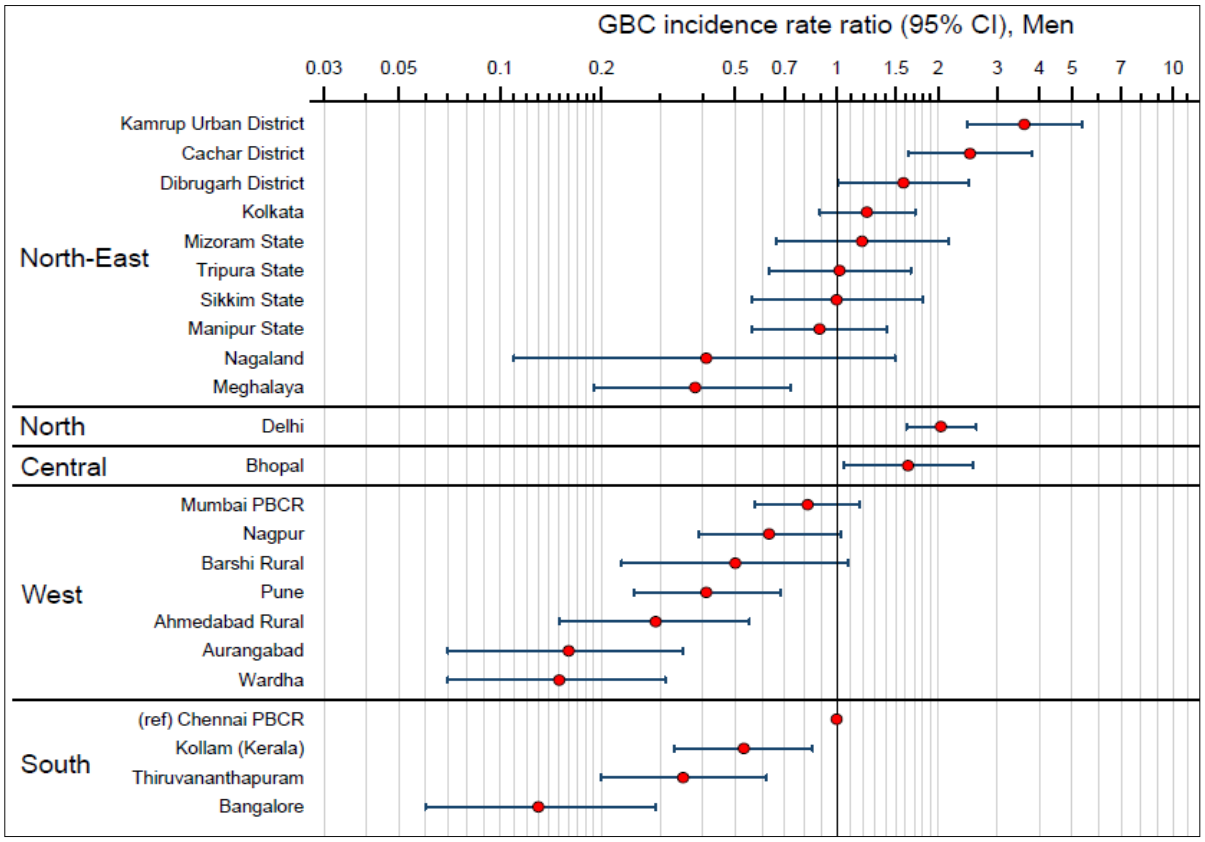
rates of GBC is observed among Japanese, Korean and Filipino population residing in Los Angeles, for both genders for the period of 1983-2007. Rates were stables in Asian countries like Japan, China and India and in European countries like Finland and Poland, Spain(34). Although the disease is declining overall, and despite the advent of new diagnostic tools combined with more radical surgery, the chance of longevity or cure is still stage dependent, with incidentally found early cancers faring favourably. The decline in GBC trends can be in part explained by the increasing number of cholecystectomies in GBC trends , however, other reasons for decline are still unknown(38).

1.2.6 India rates

In India, incidence data from National Cancer Registry Program (NCRP) indicates substantial variation by geographical region. Rates are high among the North and North-Eastern population compared to the rest of the population, with female excess in all geographical regions. Though the overall age-adjusted incidence rates of GBC in India are low, high incidence is reported in Kamrup, Cachar district, Delhi, Dibrugarh district, Sikkim state, Kolkata, Tripura, Manipur and Mizoram States **[Figure 1.7 & Figure 1.8]**. In Delhi, GBC was the fourth most common cancer (following cervix, breast and ovary) and the most common gastrointestinal cancer in women(39). Similar in Bhopal, Kolkata, and Assam, GBC is among the five leading sites of cancer in women and commonest gastrointestinal cancer in women (39). In the department of Surgical Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, about 50-60 patients with GBC are operated on every year(40). The rates for GBC are higher among women than men at virtually all ages, with gender difference decreasing slightly with increasing age. GBC is the leading cancer among digestive cancers in women in the northern Indian cities like Delhi. Evaluation of time trend for GBC has been impaired by unavailability of nationwide data in India.

However, data from low risk regions such as Pune, Chennai and Mumbai has noted decline in GBC incidence.

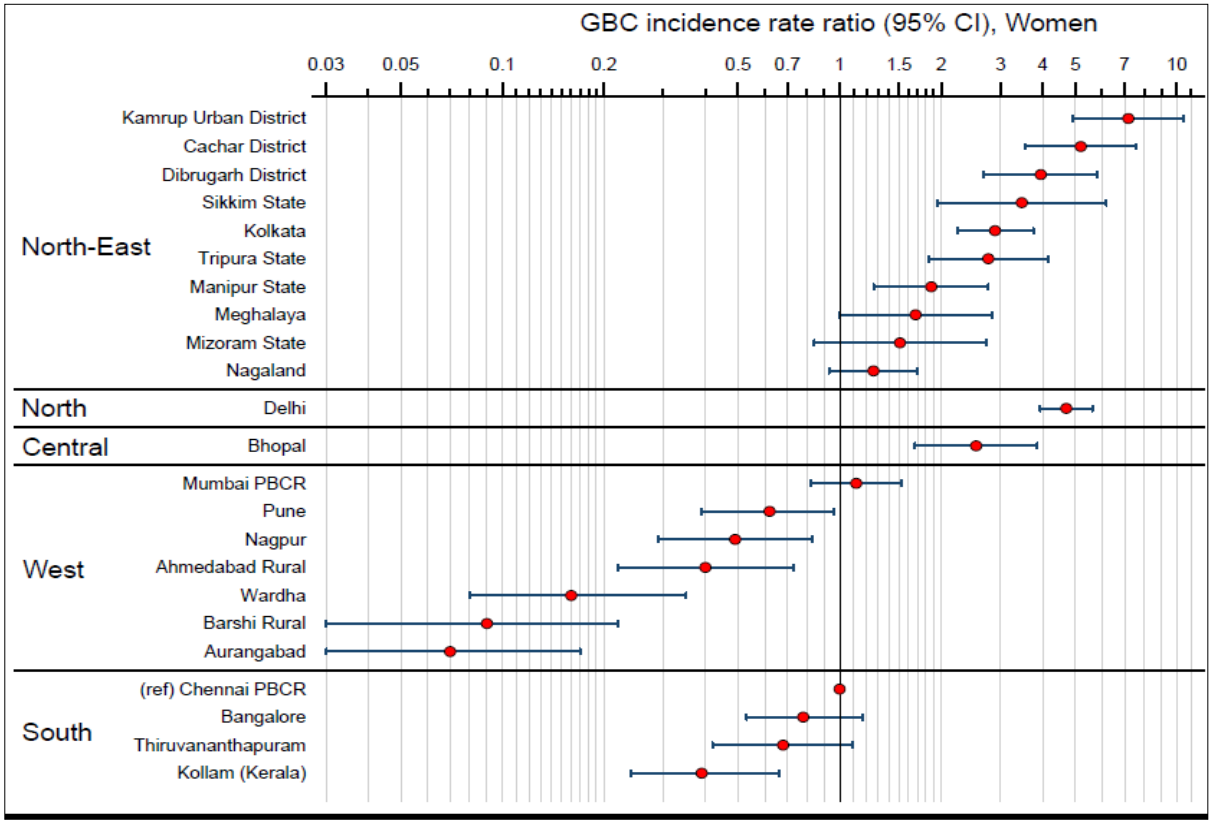
Figure 1.7 GBC incidence rates in men for selected cancer registries stratified by geographical zone



Source : NCRP-ICMR- Three year report of PBCR 2009-2011, Bangalore – 2013

AAR of GBC in Chennai: 2.05

Figure 1.8 GBC incidence rates in women for selected cancer registries stratified by geographical zone



Source : NCRP-ICMR- Three year report of PBCR 2009-2011, Bangalore – 2013

AAR of GBC in Chennai: 1.95

1.3 Etiology

GBC has generally been associated with the four Fs: (1) Fat (overweight), (2) age older than 40 years; (3) female gender; and (4) fertile. These factors certainly make women more susceptible for having GBC, but other factors also contribute to the development of GBC.

1.3.1 Gallstone

Gallstone is one of the strongest known risk factor for GBC. Moderate to strong association of gallstone and GBC have been reported by several cohort and cases control studies. It has been hypothesized that stones can lead to chronic inflammation, promoting metaplasia and adenocarcinoma by either mechanical irritation of the gallbladder mucosa or

mechanical obstruction of the gallbladder leading to cholestasis (35).Reported relative risks (RRs) from case control studies vary greatly, and this variation probably results from difference in study design and method and definitions used to collect information on gallstone. The summary of RR for history of gallstone was 4.9 [95% CI 3.3-7.4], and was 2.2(95% CI 3.3-7.4) among cohort studies and 7.1[95% CI 4.5-11.2] among case control studies(41). A high magnitude association of GBC risk with gallstone (Odds ratio: 23.8, 95% CI, 17.0–33.4] was reported in population-based case–control study in Shanghai.

In several studies, the risk of GBC has been related to the number and size of gallstone as well as duration of symptoms. Subject with stones over 3 cm in diameter have higher risk than those with stones less than 1 cm(38).

Individuals with GBC are likely to have cholesterol gallstones(42). Individuals prone to these stones tend to have "lithogenic" bile that is supersaturated with cholesterol, due to increased secretion of cholesterol or diminished secretion of bile salts and phospholipids that maintain the solubility of cholesterol. The formation of stones is also enhanced by stasis and destabilization of bile in gallbladder.

The worldwide distribution of gallstone prevalence shows a strong geographic as well as ethnic variation, and positive correlation with GBC(41) . Increased rates for both GBC incidence (per 100,000) and gallstone prevalence (%) occur in Pima Indian females (21/100,000 cancer incidence and 75.8% gallstone prevalence), North American Indian females (7.1/100,000 and 64.1%), Chilean Mapuche Indian females (27.3/100,000 and 49.4%) and East Indian females (22/100,000 and 21.6%)(32).Low -risk areas for gallstones (i.e. where prevalence is <10% among women) included African countries, but also Thailand, China, Korea, and Japan which reported high GBC incidence rates(41).

The excess risk of GBC associated with a family history of gallstones (regardless of the number and type of first-degree relatives having gallstones) is consistent with several

previous studies in various population groups. Family history of gallstones among first-degree blood relatives augment the substantial risk associated with GBC(43). These could be due to genetic factors or shared environment that increases the familial tendency to gallstones and GBC.

While GBC and gallstone share many epidemiological characteristic, the carcinogenic mechanism is poorly understood. Again, only 1-3% of patients with gallstone develop GBC, and that some tumours may arise in absence of gallstone(44). The strength of their relationship, however, is not clear. In addition, various other factors, such as age, race, lifestyle and reproductive factors may alter GBC risk.

1.3.2 Chronic Inflammation

Chronic inflammation of gallbladder in association with gallstone, infection and congenital abnormality is thought to play important role in pathogenesis of GBC. Chronic inflammation can also result in extensive calcification of the gallbladder wall. This calcified, fragile and even brittle gallbladder condition is termed as Porcelain gallbladder or calcifying cholecystitis. Porcelain gallbladder has been associated with GBC in 12-60% of patients, with the risk being greater among patients with selective compared to diffuse mucosal calcification(38). Porcelain gallbladder is more common in women than men. Although the mechanism predisposing to GBC is unclear, an inflammatory process seems likely(45). Primary sclerosing cholangitis (PSC) is a progressive autoimmune disease linking chronic inflammation to GBC. PSC patients have an increased frequency of gallbladder mass lesions, with an estimated prevalence of 3%-14% versus 0.35% in the general population. Patients with PSC and GBC tend to be younger than GBC patients without PSC; 70% of PSC/ GBC patients are less than 60 years of age, with a median age at diagnosis of 58 versus 70 years in the general population (46).

1.3.3 Infection

Persistence of infection leading to chronic inflammation, and production of toxins and metabolites with carcinogenic potentials, by infectious agent has been speculated to be involved in the transformation of the gallbladder epithelium. In addition to *Salmonella* species, certain species of *Helicobacter* (*H. bilis and H. hepaticus*) and *Escherichia coli* have also been implicated in carcinogenesis

1.3.3 *A Helicobacter species*:

Studies determining the *Helicobacter species* prevalence in GBC have raised the possible association in aetiology of GBC. PCR-based detection rates of different species of Helicobacter in biliary tract cancer vary from 0%-82.8%(47).

Helicobacter pylori (H. pylori): Findings have demonstrated the presence of *Helicobacter pylori* (H. pylori) in the bile and gallbladder of more than 75 % of patients with gallbladder cancer and more than 50 % of patients with chronic cholecystitis. *Helicobacter pylori*, one of the enteric species of *Helicobacter*, is classified by the International Agency for Research Cancer(IARC) as a Class I carcinogen because of its major causal role in gastric cancer(48). Although *H. pylori* prefers an acidic environment, such as in stomach, and may not survive in alkaline bile, molecular studies using polymerase chain reaction assay have detected bacterial DNA of *Helicobacter* species in bile fluid and tissue from patients with gallstone and GBC(38). A recent study highlights the role of *H. pylori* infection in aggravating the mucosal lesions (mucosal hyperplasia, metaplasia, and lymphoid infiltration) of the gallbladder that is considered potentially precancerous (49).The ATBC study has observed higher seropositivity to *H.pylori* protein in GBC patients (50). Studies from India have demonstrated *H.pylori* DNA in the gallbladder of a third of GBC patients and was associated with higher circulating levels of cytokines such as interleukin 1-β (IL-1β), and tumour necrosis factor-α (TNF-

α)(51). High prevalence of *H. pylori* has been observed in few studies mainly reported from India; however this association requires further findings from a large epidemiological study.

***H.bilis*:** Presence of *H. bilis* has been identified from bile specimens, with data supporting an association of bile-resistant *H.bilis* with GBC. *H .bilis* was discovered in the bile and gallbladder tissue of cholecystitis patients in Chilean population (52). A strong association was observed in the Japanese and Thai population. Reported odds ratio for GBC with *H. bilis* was 6.50 (95%CI 1.09 - 38.63) in the Japanese patients and 5.86 (1.31 - 26.33) in the Thai patients (53). In contrast, RR of 1.24 (95% CI 0.63- 2.44) was observed in meta-analysis determining role of *H. bilis* particularly in GBC. This may be due to, most studies have taken patients with gallstones as controls and this might have an influence on results as the risk of carcinogenesis could be through increase in risk of gallbladder cancer(GBC). Further observational studies are needed to ascertain the role of *H. bilis* specifically in the development of GBC using healthy individuals as controls.

Helicobacter hepaticus: This helicobacter species was discovered in 1992 as a cause of hepatobiliary cancer in the A/JCr mouse model. Studies performed on humans have revealed that *H. hepaticus* may also be a human pathogen since infection by *H. hepaticus* can be associated with cholecystitis, cholelithiasis and GBC(54).A study from Nepal has shown *Helicobacter hepaticus* infection in 82% of non-malignant gallbladders and in 87.5% of malignant cases(55). Whether *Helicobacter hepaticus* is the number one cause of the type of gallstone formation that ultimately leads to malignancy, or is itself a risk factor for the pathogenesis of carcinoma of gallbladder, is yet to be determined.

1.3.3 B *Salmonella species:*

The association between *Salmonella typhi (S.typhi)* and the risk of GBC has been reported fairly, consistently in many small observational studies. *Salmonella typhi* infection is thought to be associated with GBC by activation of serine/threonine protein kinase (AKT) and

mitogen-activated protein kinase (MAPK) pathways during infection. *Salmonella typhi* irreversibly transforms mice, gallbladder organoids, and MEFs with mutated TP53 and amplified c-MYC, which is consistent with observation in GBC patients from India(56). The overall OR for chronic *S. typhi* carrier state was 4.28(95% CI: 1.84-9.96)(57). A study from Northern India using Vi serology, presented a 7.9 times increased risk for GBC in chronic typhoid carriers(58). Another study from India demonstrated significantly higher isolation rates of *S. typhi* and *paratyphi-A* from bile, gallbladder tissue, and stones from patients with GBC. 67.3% of the GBC patients were typhoid carriers, as compared to 8.3% of the healthy population in the typhoid endemic area of North India(59). In contrast, no association was reported from a Shanghai population based case-control study(60). China is medium typhoid fever incidence country unlike India, which is classified as a high typhoid incidence country. Over 17 million people worldwide are affected by typhoid fever, the role of *S. typhi* infection in the development of gallstones and biliary tract cancer needs further clarification. Many of these epidemiological studies based on biological markers, such as serum Vi antigen or bacterial presence in bile specimens. However, PCR appears to be the most specific diagnostic tool reported in studies from India(61).

1.3.3 C *Liver Flukes*:

In China, and Thailand, bile duct cancers, particularly intrahepatic ducts, occur exclusively in populations infected with liver flukes, including *Clonorchis sinesis , Opitsthorchis viverrini*, and *Opisthorchis felineus.* Possible carcinogenic mechanisms include chronic irritation and inflammation, nitric oxide formation, intrinsic nitrosation, and activation of metabolizing genes. The association between the occurrence of Cholangio carcinoma and the presence of liver flukes has been known for about 50 years, but to be associated with GBC is unknown. From a public health perspective, thorough cooking of the fish hosts efficiently blocks infection with these parasites(**62**).

1.3.4 Reproductive and hormonal factors

The incidence for GBC is two fold higher in women, suggesting a role of reproductive and /or hormonal factors in pathogenesis of GBC. Observational studies have suggested role of endogenous hormone in promoting gallstone, increasing cholesterol, and impairing gallbladder motility, and or through a direct carcinogenic effect on gallbladder. Studies associating reproductive factors, such as increased parity and gravidity, have not a provided clear link to GBC. Furthermore, studies report conflicting associations for potential risk factors such as menarche and menopause in GBC etiology.

Age at menarche has been controversial in the etiology of GBC. Some authors have reported a late age(63,64), and others have shown an early age at menarche to be a risk factor for GBC(**65**). A prospective case control study from India has reported younger age at menarche increased the risk for developing GBC almost threefold (OR, 2.63 ; 95% CI, 1.45-96.03)**(65)**,whereas a study in the Chinese and Korean populations have reported contrary results. A Study in the Chinese population has reported women who began menstruating after age of 17 years had a 1.8 fold risk (95 % CI, 1.03-3.24) of GBC , compared with those who began menstruating at age of 13 years or younger(63). Similar findings were reported from recent cohort study of Korean women, suggesting a 37% decreased risk of GBC in women who start menstruating at the age of less than 15 years (**64**).No increased risk of GBC has been observed in women with late versus early cessation of menses**(63,64,66)**. Nevertheless, results from these studies suggest a possible role of female hormonal factor in pathogenesis of GBC. Higher and prolonged exposure to female sex hormones (estrogens and progesterone) may be a predisposing factor in development of GBC.

Multiparous women of 5 or more births report an increase risk of the GBC(65–69).The RRs ranged between 1.0 and 6.7, depending on level of parity and gravidity considered.

The reduction in risk of GBC is observed for late age at first birth. A study from china has shown that among parous women, those who had their first child before 27 years of age had higher risk of GBC relative to those who had their first child at the age of 27 or latter(63). Consistent findings have been reported from studies in India, Sweden and Italy(69–71).It is difficult to separate individual effect of parity and age at first birth , considering the high correlation between the two. However, the finding is biologically plausible, as younger age at first birth may reflect higher levels and longer exposure to oestrogen and progesterone(67).It is proposed that the lithogenicity of bile increases in pregnancy due to the effect of estrogens and progesterone hormone, predisposing to carcinoma of gallbladder(65).

Oral contraceptive use has not been materially related to GBC risk; neither were duration of use and time since first and last use. Inconsistent results were obtained for association of GBC risk with menopausal status and Hormone Replacement Therapy (HRT) use(41).

1.3.5 Overall and central obesity

Studies assessing increasing BMI as risk factor for GBC have reported conflicting findings. Over the past decades, evidence from clinical studies has addressed the possible link between excess body weight and risk of GBC, but the findings have been somewhat contradictory. Early studies found elevated but no statistically significant results between body mass index and GBC(72,73), whereas a recent study did observe a significantly increased risk(74). A meta-analysis focusing on the potential relationship between excess body weight and risk of GBC has reported that overweight and obesity were associated with 14% and 56% excess risk of GBC, respectively. Obese women had a higher risk of GBC than men did (women: RRs 1.67, 95% CI 1.38–2.02; men: RRs 1.42, 95% CI 1.21–1.66), and there was significant association between overweight and GBC risk in women (RRs 1.26, 95% CI 1.13–1.40), but not in men (RRs 1.06, 95% CI 0.94–1.20). Moreover, in cohort studies with follow-up time >10 years, overweight and obesity were strongly associated with incidence of

GBC (overweight: RRs = 1.12, 95% CI = 1.00–1.27; obesity: RRs = 1.65, 95% CI = 1.49–1.83), while only overweight was observed associated with GBC risk in cohort studies with follow-up time < 10 years (RRs = 1.52, 95% CI = 1.06–2.19). For the case-control studies, only obesity was strongly associated with GBC risk (RRs = 1.37, 95% CI = 1.10–1.71). The RRs of GBC incidence for obesity in population-based case-control studies was 1.43 (95% CI = 1.09–1.89); no significant association between obesity and GBC risk was observed in hospital-based case-control studies(75).High risk of GBC associated with obesity or overweight may reflect well-established association with antecedent gallstones or independent effects mediated by high level of endogenous estrogens, serum insulin, insulin like growth factors, or leptin in obese subjects, as well as increase in the hepatobiliary secretion of cholesterol and hypomobility of the gallbladder associated with obesity(38). Obesity may also contribute to GBC through an inflammation pathway, since fat cells secrete a large no of inflammatory mediators, such as interleukins(76).

A few studies have provided evidence of an association between central obesity and risk of GBC. European cohort study have shown the strong positive association of central obesity, measured in the form of waist to height ratio with GBC (RR: 1.56, 95% CI: 1.12–2.16 for an increment of one unit in waist to height ratio)(77).Another study was also reported that increasing levels of WHR(Waist to hip ratio) was strongly associated with excess risk of GBC , with WHR having a greater impact than BMI(78).

1.3.6 Diet and nutrition

The effect of diet on GBC is difficult to identify as the symptoms associated with gallbladder disease may induce the dietary changes. However, many epidemiological studies have implicated the role of dietary factors in development of GBC. In the multinational collaborative epidemiological study from Surveillance Aspects Related to Cancer in Humans (SEARCH) programme of the IARC , which included 169 cases and 1515 controls , the

strongest direct associations with GBC risk were for total carbohydrate intake (RR 11.3 for highest quartile versus lowest quartile) and total energy intake (RR 2.0), and inverse association for dietary intake of fibres, Vitamine B6, E and C (RR ranging from 0.4-0.5)(41). The protective effect of vegetable and fruit consumption on GBC is reported by many case control studies(69,79–81). A positive association of high fat intake with GBC risk was found in a case- control study in Karachi(69,83). Strom et al (1995) suggested non- vegetarian diet as a risk factor but they emphasized more on the cooking habit; baking/ roasting meat vs frying were a risk factor for GBC. Available evidence suggests that dietary components play a relatively small role compared with obesity, physical activity, and energy balance (38).

1.3.7 Tobacco and Alcohol

Previously published data on the relationship of alcohol consumption with GBC is inconclusive. Some studies suggested an increased risk, while others suggested that long-term alcohol use may actually play a protective role, perhaps by reducing the cholesterol saturation of bile (65). Primary care case-control study in the United Kingdom found no risk among current alcohol users (84). Contrary to that, study from Japan found significantly higher risk of cancer of the gallbladder among the male drinkers. The Hazard ratio (HR) of current male drinkers was 1.76 (0.86 -3.64) compared with non drinkers (86). However, no risk was observed in female drinkers. Recent meta-analysis has also indicated positive association between alcohol consumption and risk of GBC (RR of 2.64) (85).

Study from India found significant higher tissue nicotine concentration in gallbladder patients showings its possibly association with GBC (83). Relation of tobacco smoking and GBC has been also examined in few observational studies. A small excess risk among cigarette smokers and a suggestive dose-response relationship with amount of smoking and duration of smoking has been reported in a prospective study from Japan. Among men, the HR of current smoker was 2.27 [1.05-4.90]. HRs of those who smoked 21 cigarettes or more per day and

those with 801-1,000 cigarette-years were 3.18 [1.18-8.53] and 3.44 [1.40-8.45] respectively compared to non-smokers(86). A primary case-control study conducted in United Kingdome also observed significant risk (OR=1.61) among cigarette smokers (84). A study from eastern India has also reported significant higher risk among male smokers(87). Many studies from India have reported excess risks of GBC among tobacco chewers. An increased risk was present in any kind of tobacco chewing, whether it is Pan (lime, areca nut, catechu, betel leaf with tobacco), khaini (raw tobacco with lime) or Paan masala (processed tobacco, catechu, areca nut and lime). Studies from North India have reported RR of 2-4 in GBC patients who chew tobacco(65,68,69). Tobacco chewing is an important risk factor for GBC(87).

1.3.8 Water pollution

Evidence of the role of water pollution in GBC is limited, but some clues have emerged from studies in developing countries. A study in India reported that high levels of organochlorines in bile, including, benzene hexachloride, dichlorodiphenyltrichloroethane (DDT), aldrin , and endosulfan, were significantly higher in patients with GBC compared to patients with gallstone. This preliminary observation is suggestive of the role of organochlorines in aetiology of GBC, particularly since high rates of GBC are reported in study regions with high levels of organochlorines contaminating the river Ganges which is heavily polluted with agricultural pesticides (88).

The etiologic relation of heavy metals and their compounds has not been adequately described for carcinoma of the gallbladder. A number of heavy metals like nickel, cadmium, lead, etc. have been implicated; however, the evidence is not robust. Small Studies from India have reported that level of cadmium, chromium, and lead in bile was significantly higher in GBC patients than in gallstone patients (88,89). Presence of significant high concentration of heavy metals in GBC cancer tissue was also demonstrated in an Indo-Japan collaborative study(90).

These metals are concentrated in soil and drinking water in certain regions of India, particularly in the Gangetic belt with elevated rates of GBC. Better designed case-control studies or cohort studies looking at exposure are required .

1.3.9 Radiation

There is little evidence that ionizing radiation cause GBC. A possible excess of GBC among underground miners exposed to radon has been reported(91).

1.3.10 Anomalous Junction of Pancreaticobiliary Duct (APBDJ)

The one pathway of GBC involves, an APBDJ, a congenital malfunction of the biliary tract. In APBDJ , the premature junction of common bile and pancreatic ducts results in regurgitation of pancreatic juice in gallbladder, and consequent irritation and bile stasis that lead to an inflammatory status(35). This anomaly is rare in western countries but more apparent in Asian, particularly those of Japan origin. The association tends to occur in relatively young women, yet is not associated with stones(92). No accurate estimate of the prevalence of APBDJ in different population and the corresponding relative risk for GBC is available, but its importance in etiology of GBC in Japan is indirectly supported by results from the cohort study of 113,394 Japanese(93).

1.3.11 Heritable factors

A familial tendency to GBC has been suggested by several observational studies, although available data are insufficient for precise risk estimates. Combined data from two Italian case-control studies found an excess risk of GBC in relation to family history of GBC (OR 3.83, 95% CI 0.59-24.75)(94) . Two cohort studies in the United states and Sweden also found an association among first degree relatives (RR 2.1, 95% CI: 0.2-6.1) and specifically among parents(RR 5.1, 95% CI: 2.4-9.3) or offspring (RR 4.1, 96% CI 2.0-7.6). The summary of relative risk for family history of GBC was 4.8 (95%CI: 2.4-8.8)(92). Several epidemiological

studies have examined familial occurrence with gallstone/&GBC, suggesting underlying genetic and metabolic defects in high risk families such as American Indian.

Susceptibility gene

Despite promising leads from studies of gallstone patients, little attention has been paid to common low-penetrant susceptibility genes in the GBC(38). A substantial number of case-control studies have been reported from China and India on candidate gene single nucletode polymorphism (SNPs) for GBC susceptibility. These studies of gene variants including estrogens receptors , cholecystokinin type A receptor , adrenergic receptor, or the cholesterol hemitransporter ABCG8, increase the risk for GBC by about 2-3 fold(95). Such associations, however, have generally only been reported in single population, underpowered and need to be confirmed in larger populations of different ethnicity. A meta-analysis of 80 candidate gene variants and 173 polymorphisms among 1,046 cases and 2,310 controls revealed that most studies were underpowered or, as observed for 8-oxoguanine DNA glycosylase (OGG1, rs1052133), TP53 (rs1042522), CYP1A1 (rs1048943) and glutathione S-Transferase Mu 1 (GSTM1) Null polymorphisms, did not confirm previous association results(96).An underpowered genome-wide association study(41 GC patients and 866 controls), reported a SNP in DCC (Deleted in colorectal cancer) to be associated with GBC in the Japanese population(97). To conclude, existing candidate gene studies in GBC susceptibility have so far been insufficient to confirm any association. Future large scale epidemiological studies are requiring for studying gene–gene, gene–environment interactions and high-risk haplotypes.

1.4 Gaps in Literature

There are few studies in literature to address the aetiology of GBC from high incidence regions. Many of these studies are either underpowered to study variables or are not properly designed epidemiological studies. The precise aetiology of GBC therefore remains unclear. The role of lifestyle and environmental factors in development of GBC are not well

documented. Gallbladder anomalies and cholelithiasis are well studied risk factors for GBC. However, factors such as tobacco habits (either smoking or smokeless), dietary intake, central obesity, and family history need to be studied in adequately powered, well designed studies. Little data is available on potential susceptibility genes for GBC. These associations have generally been reported from single population and not confirmed in large studies. Recent evidence suggests the role of infection in the development of GBC. Most available evidence suggest infection with *Salmonella typhi*, *Salmonella paratyphi* and *helicobacter species* as potential risk factors. Unfortunately, most studies of infection and GBC to date have been small, have lacked properly matched controls and have suffered from lack of standardized methods.

The present thesis proposal is designed to understand the role of lifestyle related factors, gallstone and obesity along with the role of infection with *helicobacter* species in the development of GBC.

Hypothesis:

Life style factors are associated with increased risk of gallbladder cancer.

Aims and objectives:

1. **Primary:** To study the association of lifestyle related factors, gallstone and anthropometric measurement with gallbladder cancer.
2. **Secondary:** To study the association between infection with *Helicobacter pylori* and gallbladder cancer.

Chapter 2
Lifestyle and Gallbladder Cancer Risk

2.1 Introduction

The available evidence suggests that GBC is a multifactorial disease which could be attributable to various risk factors. Epidemiological studies have identified striking geographic and ethnic disparities – inordinately high occurrence in American Indians, elevated in Southeast Asia, yet quite low elsewhere in the Americas and the world. Age, female gender, congenital biliary tract anomalies, and a genetic predisposition represent important risk factors that are immutable(32). Presence of one or more gallstones represents a dominant risk factor for GBC, and the *Lith* gene predisposing to gallstone represents an important genetic risk factor(95). Many epidemiological studies have suggested that obesity is positively associated with the risk of GBC(98). Chronic infection with *salmonella typhus* and *parathphi* has been linked to elevated risk of GBC (38). Recent studies have raised the possibility that bacterial infection with *Helicobacter* species may play a role in GBC carcinogenesis. Other factors that are thought to play role in aetiology are tobacco habits, dietary intake, physical activity and past medical history (e.g. diabetes).

This chapter is mainly focused on role of lifestyle related factors in development of GBC. This chapter provides details on the following aspects of study:

- Study design, methodology and quality control assessments
- Place of birth
- Gallstone history
- Tobacco habit(tobacco smoking and smokeless tobacco)
- Anthropometric measurements
- Dietary intake
- Reproductive and hormonal factors

2.2 Study Design

A hospital based case-control study was conducted at Tata Memorial Hospital (TMH), Mumbai during the period of September 2010 to June 2015. The study has been approved by the TMH Institutional Review Board. Written informed consent was obtained from all study participants before enrolment in the study.

2.2.1 Eligibility criteria for cases

Cases were male or female patients with primary GBC (International Classification of Diseases for Oncology Version 3 [ICDO-3] site code C24) visiting TMH, Mumbai for diagnosis and/or treatment. Case eligibility criteria were:

(i) Primary GBC confirmation based on histological/cytological diagnosis from gallbladder, or unequivocal clinical and imaging diagnostic such as abdominal ultrasound and/or abdominal computed tomography/magnetic resonance imaging from gallbladder and histological/cytological finding from metastatic site

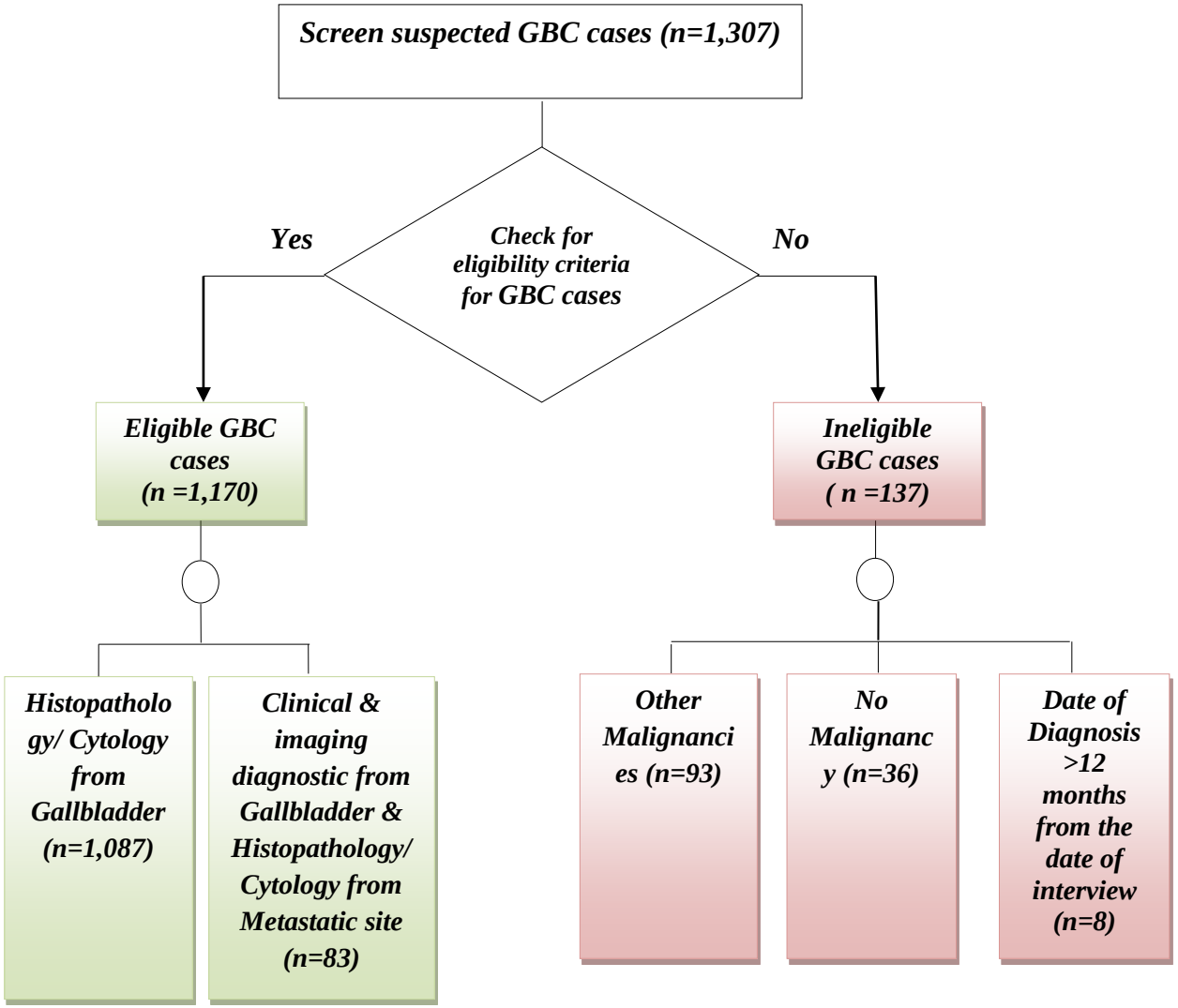
(ii) Date of diagnosis less than one year from date of enrolment

(iii) Age between 20-70 years

(iv) Resident of India for at least one year

Patient with malignancies arising from the ampulla of Vater (C24.1), overlapping lesions of the biliary tract (C24.8), unspecified regions of the biliary tract (C24.9), and any other malignancies were excluded from the study. After taking informed consent a total of 1,307 suspected GBC cases were enrolled in the study, these were further evaluated for eligibility in the study [**Figure 2.1].**Patients diagnostic details were acquired from hospital electronic medical record (EMR) system.

Figure 2.1 assessment of eligible GBC cases



2.2.2 Eligibility criteria for controls

Controls were drawn from visitors with no history of cancer accompanying patients of any cancer site to the TMH. Visitor controls aged 20-70 years were recruited from all Disease Management Groups (DMGs) of the hospital (excluding relatives of GBC cases).The proportion of controls from visitors for any individual cancer site did not exceed 20%. The majority of controls were friends, neighbours, colleagues, in-laws and spouses and other than first degree relatives [**Figure 2.2].**

2.2.3 Frequency matching of cases and controls

Controls were frequency matched to cases on age (± 10 years) and region of current residence at the time of enrolment. For region matching, India was divided into five regions: North, north-east, west, central and south.

- North (Uttar Pradesh, Bihar, Delhi, Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Chandigarh and Uttarakhand)
- North-East (Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Tripura, West Bengal, Jharkhand and Orissa)
- West (Goa, Gujarat, Daman & Diu, Dadra & Nagar Haveli, Maharashtra)
- South (Andhra Pradesh, Karnataka, Kerala, Lakshadweep, Andaman & Nicobar, Tamil Nadu, Telangana)
- Central (Madhya Pradesh and Chhattisgarh)

2.3 Data Collection

2.3.1 Questionnaire

A structured questionnaire was designed for extracting information on various lifestyle related variables. Mock and group interviews were conducted to check reliability of questionnaire. The study questionnaire underwent a series of amendments to reach the final version. Study

questions were reframed to allow better understanding of the questionnaire. The sequence of some questions was modified for effective administration of questionnaire. All these amendments resulted in achieving a higher response rate from study participants. The final version of the questionnaire consisted of demographic details, residential history, past medical history (e.g. gallstone history), tobacco habits (smoking tobacco and smokeless tobacco), dietary intake, reproductive history and socioeconomic information. All the interviews were conducted by trained research staff either in local languages (Hindi and/or Marathi) or English.

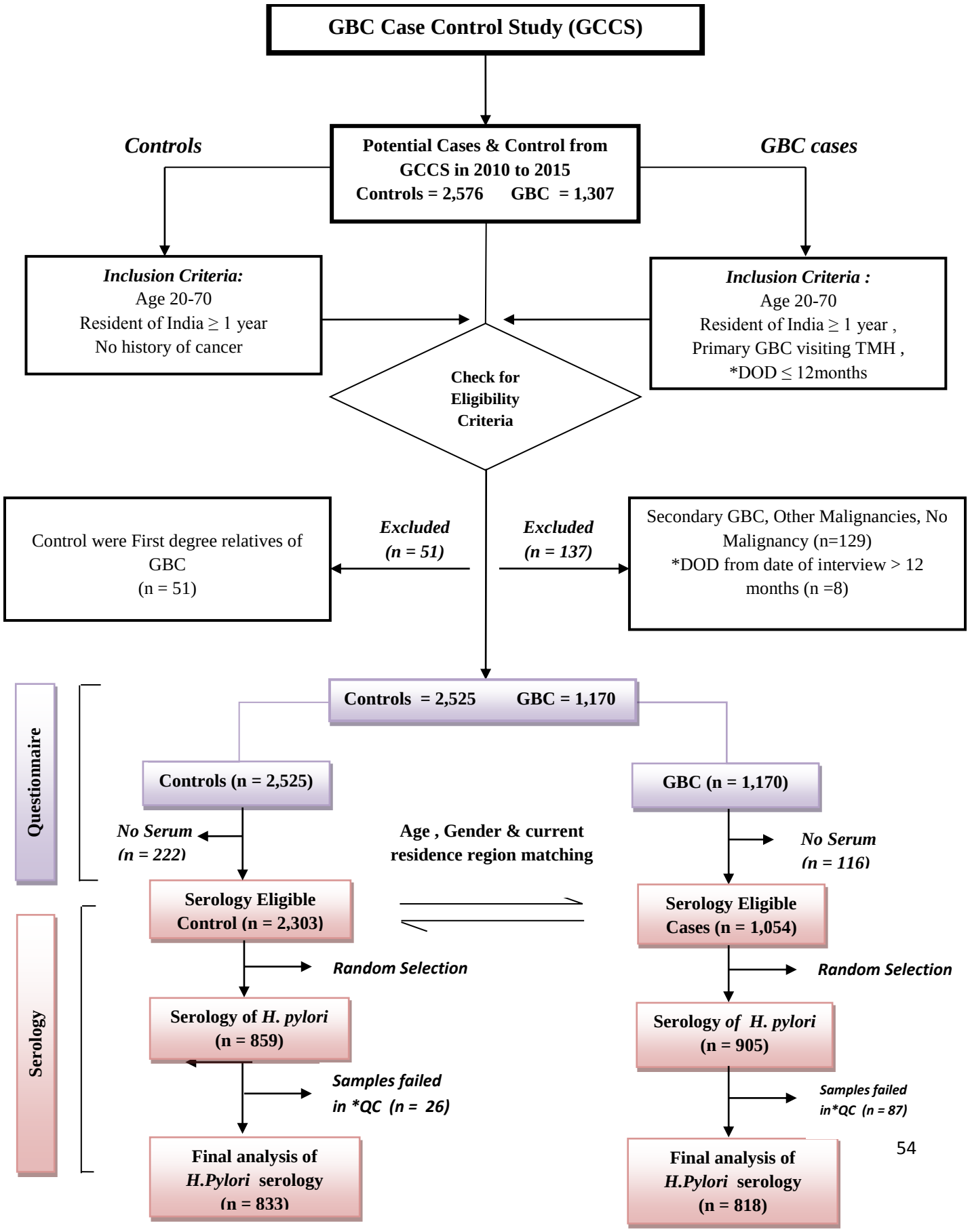
2.3.2 Anthropometric measurements

Anthropometric measurements were carried out twice using calibrated instruments, in succession and then averaged to reach a final value. A height measurement without shoes was done using a 'drop down' tape measure fixed at about 2 metres on a wall using a standardized protocol. Weight was measured using a calibrated instrument and following standardized protocol. Waist circumference (WC) was measured halfway between the costal edge and iliac crest, and hip circumference (HC) was measured as the greatest circumference around the buttocks. Circumferences were measured with a flat tape 0.5 cm wide calibrated to 1mm.

2.3.3 Blood Collection

A 10 ml blood sample was collected from each study participant and fractionated into serum and red blood cells (RBC's). The Serum sample was stored immediately at -80^0C and then transferred to -196°C in liquid nitrogen cylinder for long-term storage.

FIGURE 2.2 A FLOWCHART DESCRIBING THE ENROLMENT OF STUDY PARTICIPANTS



*DOD = Date of diagnois
*QC = Quality Control

2.4 Quality Assessments

2.4.1 Preparation of instruction manual for questionnaire based data collection

In order to ensure the quality of data collection by the research staff, an instruction manual was developed. The manual contains comprehensive guidelines for data collection [13].

2.4.2 Preparation of instruction manual for data entry in electronic database

A comprehensive instruction manual was prepared to ensure the consistency in data entry [14]. Term of codes for both qualitative and quantitative data were pre-decided and enlisted in manual. An adequate comprehensive error localization function was defined for correct data entry by the data entry typist.

2.4.3 Quality or logical check on electronic data

Quality /logical checks were introduced in the electronic database to minimize transcription and typing errors. Data cleaning was commenced once data was entered twice in the electronic database. Double data entry was carried out by two different data entry typists to capture data entry errors or missingness. Errors identified between the two entries were corrected using a hard copy of the questionnaire as reference [**Table 2.1**], if any error occurred while entering the data.

Table 2.1 Example of corrected entries for "gallstone history" between data entry 1 and 2

Study ID	First time data entry	Second time data entry	Corrected data entry
810299	1	2	1
810218	2	88	88
810503	1	2	2
841288	2	2	2

2.4.4 Quality control monitoring for data collection and data entry

Regular examinations were carried out on all questionnaires for human errors and missingness in data after conducting the interviews and after the data were entered in the electronic database. Completeness of data assessment was accomplished at three different levels: data were first examined by the interviewer, immediately after taking the interview, second by the project co-coordinator, on the day following the interview, and by the data entry typist before entering the data. Weekly meetings were conducted to understand and resolve issues of data collection and data recording in electronic database. Training programs were conducted every quarter so as to ensure the quality of interviews and data entry typist.

2.4.5 Reproducibility of questionnaire

An abbreviated questionnaire was designed to evaluate the reproducibility of questionnaire. A reproducibility check was performed by re-interviewing 253 study participants. The abbreviated questionnaire contains unchanging variables (for recent time) such as height, age at menarche, current residential region, number of pregnancies, and vegetarian /non-vegetarian status. This abbreviated questionnaire was administered after a minimum interval of 7 days after administration of the first interview. Smoking, chewing, alcohol drinking and use of hormone releasing contraceptives showed 100% reproducibility. Other important variables such as completed age, height, number of pregnancies have shown more than 80% correlation in two measurements [**Table 2.2**].

Table 2.2 Reproducibility of measured variables

Variable	Study Mean (Reproducibility Mean) N=253	Coefficient of Correlation (%)
Age	46.77 (47.07)	92.29
Number of Pregnancies	4.19 (4.12)	85.12
Height	156.05 (157.32)	96.44
Current Residence	NA	90.64
Education	NA	87.15
Age at diagnosis of gallstone	46.12 (46.11)	90.42

2.4.6 Quality control of anthropometric measurements

Regular calibration process was performed on weighing balance, measuring tape, wall mounted stadiometer for precise anthropometry measurements. Weighing balance, measuring tape and wall mounted stadiometer were calibrated twice a year using an unused weighing balance which was used as standard and a difference of ±1kg was considered acceptable. Similarly an unused measuring tape was used to calibrate the measuring tape and wall mounted stadiometer and a difference of ±1cm was considered acceptable.

2.5 Exposure Assessment

2.5.1 High risk and low risk region

In order to quantify the geographical variation in GBC incidence, we divided Indian states and territories into high and low risk regions using incidence rates extracted from population based cancer registries (PBCRs). States were considered to be in high risk region if the state PBCR reported average age-adjusted rates of greater than 5.0 per 100,000 persons. All other PBCR

states were considered as low risk region. In states with no cancer registry, we used the minimum age adjusted incident rate (MAAR) provided by the Atlas of Cancer in India, to classify the states into high and low risk regions using the same threshold of incidence rate. Thus Bihar, Delhi, Himachal Pradesh, Punjab, Chandigarh, Rajasthan, Uttarakhand, Uttar Pradesh, Assam, Tripura, Sikkim, Jharkhand and West Bengal were categorised into high risk regions (mainly north and north Indian states) and the remaining states classified as low risk regions. Effect of length of residence in a high-risk region, was evaluated by stratifying study participants into the following mutually exclusive categories:

- Never lived in a high-risk region (reference)
- Lived for a minimum of 1 year but less than 20 years in a high-risk region
- Lived for 20 and more than 20 years but less than a lifetime in a high-risk region
- Lifetime (If study participant has lived the entire course of his/her life in a high-risk region)

2.5.2 Gallstone history

Ascertainment of gallstone status was based on self reports of gallstone history by study participant. Following the two different definitions were used for categorizing study participants on their gallstone history:

- **Self reported gallstone:** as reported by study participants i.e. either present / not present
- **Self reported gallstone using stringent definition:** Gallstone history was ascertained using definition of self reported gallstone; however those gallstones diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to

the date of interview for controls were categorized as "not present". Otherwise was categorized as "present".

This association was further evaluated for duration of gallstone. Gallstone duration was determined using "age at the time of gallstone diagnosis" and "age at the time of interview". Gallstone duration categories for self-reported gallstone were: gallstone not present (reference), ≤1 year, 2-4 years and ≥ 5 years. Categories for "self reported gallstone using stringent definition" were gallstone not present (reference), 1-2 years, 3-4 years and ≥ 5 years.

2.5.3 Tobacco habits (tobacco smoking /smokeless tobacco)

The structured questionnaire sought detailed information on consumption of tobacco both smoking and smokeless tobacco. Tobacco questions included a screening question to identify regular smokers/chewers. The definition of regular smokers/chewers for current study is those who smoked/chewed tobacco for at least once a week for at least six months in his / her lifetime. Those responding positively to the tobacco smoking/chewing screening question, were interviewed for detailed information on tobacco consumption such as age at which consumption commenced and pattern of tobacco consumption.

Tobacco association was evaluated using different categories of tobacco consumption: "Any type of tobacco smoking", "bidi smoking", "any type of smokeless tobacco", "tobacco *quid* " & "betel leaf *quid* with tobacco". "Any type of smoking" included different forms of smoking such as cigarette, bidi (thin cigarette filled with tobacco flake and wrapped in a tendu leaf tied with a string at one end), cheroot or stogie (cylindrical cigar with both ends clipped during manufacturing), cigar (tightly-rolled bundle of dried and fermented tobacco), water pipes/hookah (single or multi-stemmed, often glass-based instrument for smoking in which the smoke is cooled by water), roll your own

(cigarette made from loose tobacco and rolling papers), chuttas (coarsely prepared cheroots), dhumti (conical cigar made by rolling tobacco leaf in the leaf of another plant), hooklis (clay pipe),chillum (straight conical pipe with end-to-end channel, traditionally made of clay). However, detailed analysis on daily consumption, duration of usage, and cumulative years of consumption was evaluated only for "bidi smoking" as usage of all other forms of smoking tobacco was limited in study population **[Table 2.9].** Bidi smoking dose level was evaluated by conducting categorical analysis of number of bidi smoked per day (non-smokers (reference), 1-14 bidi /day, 15-29 bidi/day, >29 bidi/day) and bidi smoking duration in years (non-smokers (reference), 1-10 years, 11-20 years, >20 years of bidi smoking consumption). Cumulative years of bidi smoking was computed to evaluate effect of intensity of smoking, by multiplying number of bidi smoked and bidi smoking duration in years, which was then categorized into non-smokers (reference), 1-199, 200-400, >400. "Tobacco *quid"* intake was evaluated to study the effect of tobacco chewing on GBC risk. "Tobacco *quid"* is the combination of tobacco, areca nut, slaked lime, and catechu. The dose relationship was assessed by categorizing the study participant on their daily intakes (non-tobacco chewers (reference), 1-5 times, >5 times in day) and duration of "tobacco *quid* intake" (non-tobacco chewers(reference), 1-19, >19 years) on development of GBC. Relationship between "betel leaf *quid* with tobacco" intake and GBC was also explored in current study. "betel leaf *quid* with tobacco" is the combination of betel leaf, areca nut, slaked lime, and catechu with tobacco. Effect of "betel leaf *quid* with tobacco" was further studied for intake (non-tobacco chewers(reference), 1-5, > 5 times) and duration (non-tobacco chewers(reference), 1-19, >19 years independently). Analysis was also carried to estimate cumulative effect of all different forms of smokeless tobacco (used by study participants in

present study) on GBC risk by computing new variable "any type of smokeless tobacco". Cumulative effect of "any type of smokeless tobacco" was studied further for dose response and linear trend effect on GBC using categories used in the analysis of "tobacco *quid*". "Any type of smokeless tobacco" included intake of smokeless tobacco in any form: tobacco *quid*, betel leaf *quid* with tobacco, Pan masala (commercial preparation containing areca nut, slaked lime, catechu and condiments with powdered tobacco), Manipuri tobacco (tobacco with slaked lime, finely cut areca nut, camphor and cloves), tobacco and slaked lime (khaini), snus (Swedish snuff), bajjar/tapkir (dry snuff), Lal Dant Manjan (a red-coloured tooth powder. Traditionally, which contained tobacco but after the passage of a law in date banning the use of tobacco in dental care products, the listing of tobacco as an ingredient was stopped), gul(pyrolysed tobacco product), gudhaku (paste made of tobacco and molasses),gutka (dry mixture of crushed areca nut, tobacco, catechu, lime, aromas , flavouring and other additives), mawa (mixture of shaving of areca nut, scented tobacco, lime), mishri (roasted, powdered preparation made by baking tobacco on a hot metal plate until it is uniformly black), and creamy snuff (commercial preparations of tobacco paste marketed in toothpaste-like tubes which are advertised as possessing anti-bacterial activity and being good for the gums and teeth).

2.5.4 Anthropometric Measurements

Weight, height, WC, HC and waist to hip ratio(WHR) were analysed using sex-specific tertiles and/or quartiles with the lowest tertile/quartile as reference category.

2.5.4 A Height and weight:

For men, height was grouped into ≤ 160 (reference), 161-164, 165-168 and ≥169 cm and weight was categorized into ≤ 53 (reference), 54-62 and >62kgs. For women, height was grouped into

≤150 (reference), 151-155, 156-160 and ≥161cm and weight was categorized into ≤ 60 (reference), 61-65 and >65kgs.

2.5.4 B Body mass index (BMI):

BMI, a measure of general obesity was calculated by dividing weight in kilograms by the square of height in meters. Predefined categories of BMI (Underweight(<18.5), Normal (18.5-24.9) (reference), Pre-obese (25.0-29.9) and obese (≥30)) based on WHO criteria for world population(99) were used for analysis.

2.5.4 C Central obesity:

WC, HC, and WHR were used as a measure of central obesity. WC was measured halfway between the costal edge and iliac crest and HC was measured as the greatest circumference around the buttocks(100). WHR was calculated by waist circumference by hip circumference.

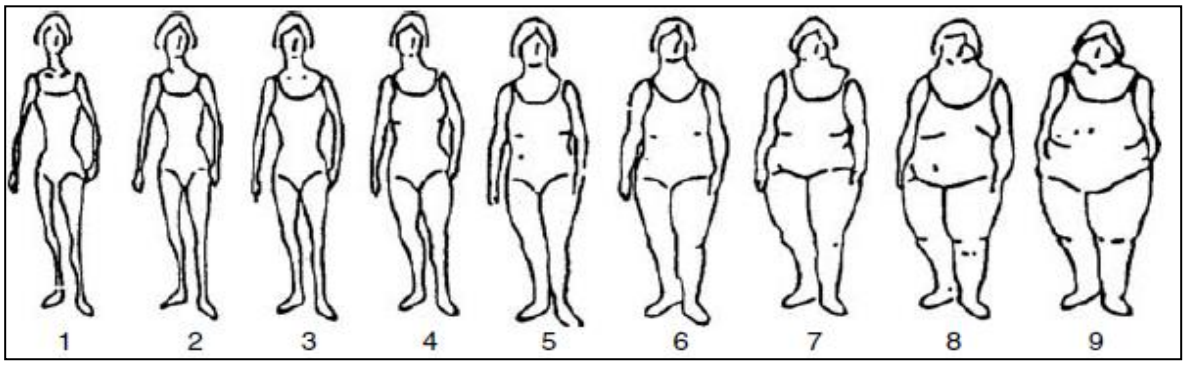
WHR is measure of body fat distribution (abdominal vs hip). For men, WC was grouped into ≤83 (reference), 84-92, and ≥93 cm; HC was categorized into ≤ 90 (reference), 91-99 and >100cms and WHR was classified into ≤0.90, 0.91-0.96, ≥0.97. For women, WC was grouped into ≤79 (reference), 80-85, and ≥86 cm ; HC was categorized into ≤ 90 (reference), 91-99 and >100 cms and WHR was classified into ≤0.84(reference), 0.85-0.94,≥0.95.

2.5.4 D Body size pictogram:

Using a pictogram**[Figure 2.3],** study participants were asked to chose among nine illustrations of body size ranging from very lean to obese which they felt represented by their body size most accurately at each of three time intervals (at 10 years, 20 years and at the time of enrolment). Similar pictograms have been used and validated in many epidemiological studies(101). Body size pictograms were given a score of 1 to 9 ranging from very lean to severely overweight

which were then categorized into < 3 (reference), 3-4, ≥5. Using study data, the correlation between BMI calculated using measures at the time of enrolment and body size score using Spearman rank correlation was found to be 0.55. Correlation coefficients were similar between cases and controls.

Figure 2.3 body size pictogram



2.5.5 Dietary intake

Information on diet was assessed with the 77 –dietary item semi-quantitative food frequency questions listed in main structured questionnaire. Study participants were asked about consumption of dietary items listed in a food frequency questionnaire, which they were typically consuming, prior to the diagnosis of GBC for cases and one year before to the date of interview for controls (Never, per week, per month, per year).For selected vegetables and cereals that were known to be consumed both raw and cooked, study participants were asked separately about intake of each. Foods were classified into groups **[Table 2.3]** and total intake was computed. The sum of the weekly frequency of intake of food items into food group was distributed into quartiles. Food groups that did not vary in intake were divided into median, based on consumption of the control study population. Odds ratios (ORs) and corresponding 95% confidence intervals(95% CIs) for each dietary item under consideration were estimated using

unconditional logistic regression model after adjusting for potential confounders using the lowest quartile of consumption as reference. Models assessing fruit and vegetable intake were controlled for any meat consumption, while models assessing meat products were controlled for consumption of any fruit. Birth region specific (High risk region vs Low risk region) analysis was carried for fenugreek, fresh fish and mustard oil consumption to examine the effect of regional variation in dietary intake with GBC. The study also evaluated the effect of lifelong vegetarianism status against that of non- vegetarianism on development of GBC. Study participants following practice of plant-based diet without the inclusion of eggs and meat one year before to the date of interview for controls and before the diagnosis of cancer for cases were categorised as "vegetarian". Study participant was considered vegetarian if he/she reported eating at most 3 times in a year, or has eaten mistakenly or eats bakery products that contain egg. Study participants following practice of any meat consumption were categorised as "non-vegetarian". Study participants were further interviewed for the number of years they were following these diets and the duration of diet practice was quantified. Lifelong vegetarians are those practiced vegetarian diet over their entire course of life (one year prior to interview for controls and before the diagnosis of cancer for cases).

Table 2.3 Listed dietary items and their respective food classes

Food groups	Dietary items
Any fruit	Banana, Apple, Mango, Melon, Watermelon, Pear, Grape, Citrus fruits (Orange/ Sweet lime)
Citrus fruits	Orange/ Sweet lime
Non -citrus fruits	Banana, Apple, Mango, Melon, Watermelon, Pear, Grape
Any vegetable	Raw Vegetables : Cucumber, Carrots, Radish, Onions, Garlic, Tomatoes, Beet root
	Cooked vegetables: Potatoes, Onions, Garlic, Tomatoes, Ladies fingers , Brinjal /Egg plant, Bitter Gourd, Carrot, Radish, Turnip, Beetroot, Cauliflower, Cabbage, Pumpkin, Spinach, Colocasia, Bottle gourd, Beans, Ivy Gourd, Fenugreek Leaves, Drumstick, Cowpea leaves, Mixed vegetables
Leafy green vegetables	Cooked vegetables: Spinach, Fenugreek Leaves, Cowpea leaves
Root vegetables	Raw Vegetables : Carrots, Radish, Onions, Garlic, Beet root
	Cooked vegetables: Potatoes, Onions, Garlic, Carrot, Radish, Turnip, Beetroot, Colocasia
Fruit vegetables	Raw Vegetables : Cucumber, Tomato
	Cooked vegetables: Tomatoes, Ladies fingers , Brinjal /Egg plant, Bitter Gourd, Pumpkin, Bottle gourd, Beans, Ivy Gourd, Drumstick
Cruciferous vegetables	Cauliflower, Cabbage
Tomato	Raw as well as cooked tomato
Onion & garlic	Row as well as cooked onion /garlic
Any spice	Green chillies, Red chillies , Turmeric , Garam Masala which is a blend of ground spices (whole / powder / grind form/ raw /cooked) , Pickles (Any pickle including of prawn pickle)
Any cereal	Rice ,Maize, Bajara, Jowar, Flaked rice, Chapatti, Bread
Any pulses	Lentils, Green gram, Bengal gram, Black gram, Red gram, Soya ,Moth beans,
Fenugreek	Whole / powder / grind form/ raw /cooked fenugreek
Pickle	Any pickle including of prawn pickle
Any dairy product	Milk, Butter, Cheese, Buttermilk, Curd/yogurt, Cottage Cheese, Condensed Milk
Any meat	Mutton, Beef, Chicken, Fresh fish, Dry fish, Egg
Mutton	Mutton(the flesh of sheep)
Chicken	Chicken
Fresh fish	Fresh fish
Dry fish	Dry fish(fish cured with dry salt and thus preserved for later eating)

The questionnaire elicited detailed information on 11 different types of cooking oil. Study participants were asked about their household monthly consumption of cooking oil (grams, kilograms). This information was then linked to data on the total number of family members in each household, and per member consumption was calculated. Percent consumption was calculated for cooking oil use among study participants. Detailed analysis was conducted only for commonly used cooking oil (found to have percent more than 15%). Based on monthly consumption among controls, cooking oil intake was divided into tertiles. Odds ratio and corresponding 95% confidence interval for cooking oil under consideration was estimated using unconditional logistic regression model after adjusting for potential confounder. No and /or the lowest category of oil consumption was used as the reference category.

2.5.6 Reproductive and hormonal factors

Reproductive history included number of full–term pregnancies, age at menarche, and menopause, hormone releasing contraceptive usage, and spontaneous and induced abortion.

Total number of pregnancies was defined as never (reference) and ever (inclusive of abortion, miscarriage, still birth and full-term pregnancies). Reported total number of full-term pregnancy was categorized into 1 (reference), 2, 3 and ≥4. Age at first full-term pregnancy was categorized into <20 (reference), 20-21, 22-23, 24-25 and ≥26 years. Age at last full-term pregnancy was grouped into women who had their last pregnancies at age ≤24 (reference), 25-29, 30-34 and ≥35. Duration since last birth (in years) was categorized into ≤10 (reference) , 11 to 20, 21 to 30, >30.

Age at menarche was grouped into age ≤12 (reference), 13–14, and 15-20 years. Age at menopause was grouped into ≤46 (reference),47-48,49-52,≥ 53 years .

Any intentional termination of fetus was classified as “Induced abortion” whereas spontaneous loss of fetus before the 20th week of gestation, or stillbirth by natural means was termed as “Spontaneous abortion” in the study. Number of induced and spontaneous abortions were grouped as 0 (reference), 1 and ≥2.

Oral contraceptive use was grouped into never (reference) and ever. Ever oral contraceptive users are those who had used oral contraceptive at least once in their life time. Age of oral contraceptive use was grouped into ≤24 (reference), 25-29 and ≥30 years. Total duration of oral contraceptive used measured in years was categorised as never (reference), < 13, 13-49 and > 49 months.

2.6 Statistical Analysis

Estimates were calculated as crude and adjusted odds ratios (95% CIs) for birth place, anthropometric measurements, gallstone history, tobacco consumption, dietary intake and reproductive factors (102). Analyses were performed with and without stratification on gender. Unconditional logistic regression models were adjusted for potential confounders such as age, current residential region (North, North-East, West, Central and South), education (Less than 5 years of schooling, ≥5 year of education), gender, tobacco chewing and tobacco smoking (yes/no), gallstone history (not present/ present), and waist to hip ratio (continuous variable). Female specific models were additionally adjusted for number of full term pregnancies (continuous variable). Effect of gallstone on gallbladder cancer was evaluated by computing odds ratio and their 95% CIs using two definitions of gallstone: “self reported gallstone” & “self reported gallstone using stringent definition”. Role of tobacco in development of gallbladder cancer was evaluated using different categories of the tobacco usage: “Any type of tobacco smoking”, “bidi

smoking", "any type of smokeless tobacco", and "tobacco *quid*", "betel leaf *quid* with tobacco" chewing Models estimating risk of tobacco chewing were adjusted for tobacco smoking ,while models assessing tobacco smoking were adjusted for chewing . For estimating ORs for WHR, WC, HC instead of WHR; BMI in kilogram as continuous variable was entered in the statistical model. BMI was adjusted as continuous variable instead of WHR in pictograph based body size analysis for body size at age 10, age 20 and current age. Models assessing fruit and vegetables intake were controlled for any meat, while models assessing meat products were controlled for any fruit. Quantitative birth place region specific analysis was carried on dietary variables such as fenugreek, fresh fish and mustard oil consumption to study the residual effect of residential region on these variables. Considering the strong correlation between parity and age at first full live birth among controls (r= -0.40), their joint effect on GBC was examined. Test for linear trend for ordered variables were performed by assigning the score j to the j^{th} exposure level of a categorical variable (where j = 1, 2 . . .) and treating it as a continuous predictor in unconditional logistic regression. Analysis was performed on non –missing values and study participants with missing value for one or more of the variables in statistical model were eliminated from analysis. All analyse were performed using the statistical package Stata version 12.0 (103).

2.7 Results

A total of 1,170 GBC cases and 2,525 controls were included in the analysis. GBC diagnosis was based on histopathological and cytological confirmation in 92.2% of cases. Of the tumours, with histopathological confirmation, over 90% were adenocarcinomas. Table 2.4 shows selected characteristics of cases and controls with respect to age, gender, current residential region, education, and birth region and gallstone history. The mean age at enrolment of cases and

controls was 49.85 and 46.03 years respectively **[Table 2.4]**. GBC was more common in women accounting for 67.52% of total cases. Greater percentage of GBC cases were from north (45.64%) and north-eastern regions (13.33%) of the country. 81.82 % of GBC cases were born in high risk region compared to 16.18% born in low risk region.

2.7.1 Place of birth

The ORs of developing GBC for those reporting birth in a high risk region was observed to be 4.82 (95%CI: 3.87-5.99) compared to those born in low risk region, after adjusting for potential confounders. The observed risk was much stronger in women (OR=6.04; 95%CI: 4.52- 8.07) than in men (OR=3.17; 95%CI: 2.23-4.50). Risk increased with increasing duration of residence in a high-risk region. The risk was maximum for those who always lived in the high risk region compared to those who never lived in high risk region (OR =5.58; 95%CI: 4.42-7.05).Study participants born in high risk regions reported high susceptibility of GBC even after migration from high to low risk region (OR= 1.36 ; 95% CI: 1.02-1.82) **[Table 2.5].**

2.7.2 Gallstone history

Self reported gallstone:

The overall prevalence of self reported gallstone in the 2,525 controls was 2.39%. The ORs associated with gallstone using this definition were 28.94(95%CI: 21.55-38.86), 30.89(95%CI: 16.62 -55.22), and 29.40(95%CI: 20.65-41.86) for all, men, and women, after adjustment of potential confounders **[Table 2.6, 2.7, 2.8]**. GBC risk was substantially decreased with increasing gallstone duration (≤1, 2-4, and ≥5 years). Very high ORs were observed for GBC related to gallstone history diagnosed within a year or year prior to GBC ($OR_{\leq 1}$=156.77; 95% CI: 80.00-307.20) for all study participants **[Table 2.6, 2.7, 2.8]**.

Self reported gallstone using stringent definition:

To assess the possibility of concomitant detection of GBC and gallstone, study participants with gallstone diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to the date of interview for control were categorized as "not present". Otherwise was categorized as "present". The ORs associated with gallstone history using stringent definition were 6.10(95%CI: 4.34-8.56), 10.29(95%CI: 4.67-22.66), and 5.16(95%CI: 3.51-7.59) for all, men, and women respectively after adjustment of potential confounders **[Table 2.6, 2.7, 2.8]**. Highest association was observed with gallstone diagnosed within 1-2 years before GBC occurrence ($OR_{\leq 1}$=16.60; 95%CI: 8.64-31.89), while for those with diagnosis of gallstones for 5 year or more before GBC occurrence was 1.93 (95%CI: 1.15-3.24) for all study participants.

2.7.3 Tobacco habits (Tobacco smoking and smokeless tobacco)

The habit of tobacco smoking and chewing was more common in cases (12%, 25%) than controls (10%, 20%). Among smokers, bidi smoking was the dominant form of smoking **[Table: 2.9].**Use of smokeless tobacco is more prevalent in study population. Tobacco *quid* and betel leaf *quid* were commonly used form of smokeless tobacco among study participants **[Table 2.10]**. The risk of GBC was found to increase with bidi smoking duration ($OR_{>20\ years}$ =2.13; 95%CI: 1.24-3.65) and number of bidi smoked/day ($OR_{>29bidi/day}$ =1.83, 95% CI: 0.80-4.20). The GBC risk of >400 cumulative years of bidi smoked was 2.41 fold higher compared to non-bidi smokers **[Table 2.11]**. GBC risk was also related to daily frequency of any type of smokeless tobacco use (*P trend* = 0.006) and was 1.47 fold for using smokeless tobacco at least 5 times or more daily **[Table 2.12].** Among chewers, length of time, "tobacco *quid*" chewing have strong

effect on GBC risk ($OR_{>19\ years}$ =1.43, 95%CI: 1.05-1.94). GBC risk was significantly elevated among "tobacco *quid*" chewers who chewed more than 5 times /daily ($OR_{>5\ times\ /day}$ =1.60, 95% CI: 1.17-2.20). Similar trend of increase risk was observed among "betel leaf *quid* with tobacco" chewers **[Table 2.14].** A risk of 1.6 was observed for GBC among "betel leaf *quid* with tobacco" chewers for >19 years ($OR_{>19\ years}$ =1.60, 95%CI, 1.08- 2.37). Analysis also showed, increased in risk for about two times for those who consumed at least 5 or more times consumed "betel leaf *quid* with tobacco" ($OR_{>5\ times\ /day}$ =2.61, 95% CI, 1.40-4.88).

2.7.4 Anthropometric Measurements

WC, WHR, body size at age 10 and 20 were significantly associated with higher risk of GBC in both genders. Higher waist circumference had an increased risk of GBC. The WC found to be associated with increased risk of GBC in men ($OR_{WC\ \geq 93}$= 2.84, 95%CI: 1.51-5.34) and in women ($OR_{WC\ \geq 86}$= 2.14, 95%CI: 1.46-3.14). A higher WHR (men, ≥0.97; women ≥0.95) was associated with a 1.93 fold (95% CI: 1.30-2.86, *P trend* 0.001) in men and 4.68(95% CI: 3.20-6.86, *P trend* ≤0.001) in women, making possible association with cancer of gallbladder. Higher risk was seen for men study participants with low BMI (<18.5) and was $ORs_{World\ population}$: 2.81, 95% CI: 1.74-4.53. Similar association was observed in women; with low BMI (<18.5) and was $ORs_{World\ population:\ 3.43}$, 95% CI 2.35-5.01. An inverse relationship has been observed between weight and BMI with GBC risk across all categories [**Table 2.15, 2.16**]. The risk of gallbladder cancer decreased with increasing height in both sexes [**Table 2.15, 2.16**]. The ORs in highest quartile compared to lowest quartile were 0.63(95% CI: 0.41-0.96) and 0.71 (95% CI: 0.42-1.21) for men and women respectively.

Body sizes at ages 10, 20 and at enrolment were evaluated for their association using pictogram illustrations with GBC risk after adjusting for BMI. Body size at age 10 and 20 found to be positively associated with GBC in both genders; even after adjusted for potential confounders [**Table 2.15, 2.16**]. On the other hand, body size at the time of enrolment was shown to decrease the risk of men (OR = 0.65; 95% CI: 0.39–1.07) and women cancer (OR = 0.50; 95% CI: 0.33-0.74) even after adjusting for BMI. This association was consistent with other measures of overall obesity such as weight and BMI.

2.7.5 Dietary intake

A protective association from GBC was observed with increasing intake of any fruit, citrus and non citrus fruits, any vegetable, leafy green vegetables, fruit vegetables, tomatoes, onion and garlic **[Table 2.18]**. Higher intake of any cereals intakes was found to be protective against GBC **[Table 2.18]**.

Study observed no association between all spices together and GBC. Habit of eating fenugreek was associated with elevated risk of GBC ($OR_{\text{Highest quartile}}$ = 1.91, 95% CI: 1.50-2.43).

Table 2.20 shows the relationship between intake of dairy products and GBC risk. No statistically significant association was observed between intake of dairy products and GBC ($OR_{\text{Highest quartile}}$=1.01, 95%CI: 0.78-1.29).

Statistically significant association was not observed for lifelong vegetarianism practice and GBC **[Table 2.17].** Similarly, no significant association was observed for "any meat", "mutton", and "chicken" and consumption with GBC risk. High consumption of fresh fish was associated with increased risk of GBC ($OR_{\text{Highest quartile}}$ =1.41, 95% CI: 1.10-1.20). Consumption of dry fish on the other hand found to be protective against GBC ($OR_{\text{Highest quartile}}$= 0.75, 95%CI: 0.61-0.92).

High consumption of mustard oil was observed to increase risk of GBC(OR $_{\text{Highest tertile}}$ = 3.41, 95%CI:2.73-4.25).Mustard oil was the most commonly consumed oil among study participants**[Table 2.21]**. On the other hand, protective associations were observed for sunflower oil, soya oil and animal ghee consumption **[Table 2.22]**. Multivariate analysis was not conducted for palm, ginger, cauliflower, coconut, and groundnut oil and mix vegetable oil due to their low percent consumption among study participants. When oil consumption was adjusted for each other in model, soya oil could not retain its significant association, while the results for other oil consumption did not alter significantly (data not shown).

Risk with mustard oil consumption was even persistent in birth region specific analysis **[Table 2.23]**. While, the risk remains elevated for fenugreek and fresh consumption in birth region specific analysis, these risks were no longer significant.

2.7.6 Reproductive and hormonal factors

Number of full term pregnancies was positively associated with GBC after adjusting for potential confounders. Compared with women who had one child, those having four and more children had 2.34-fold risk (95% CI: 1.46-3.74) of GBC. Given the strong association between parity and age at first birth, their joint effect was examined on GBC **[Table 2.25].** The risk of GBC increased with increasing parity, regardless of age at first full term pregnancy, but was highest for women having 3 and more children, with the first birth at the age of 24 years and older (OR=2.57, 95% CI 1.11-5.95).GBC risk also increased with increasing parity regardless with age at menarche , but the highest risk was seen for women having four or more children , with age of menarche being 16 years or older(OR=2.68, 95% CI: 1.61-4.45). No association was observed among women who were ever pregnant as compared to women who were never pregnant **[Table**

2.24]. A late age at last birth was also associated with non-significant but increased GBC risk (OR $_{\leq 24}$ vs OR $_{\geq 35}$=1.49, 95% CI: 0.97-2.30). An increasing trend was seen with increasing age at last full-term pregnancy with the risk of GBC even after adjusting for total number of pregnancies (*P trend*=0.028). Longer the "duration since last live birth" was significantly protective in GBC ($OR_{>30}$ =0.44, 95%CI: 0.22-0.87). Women who began menstruating at or after age the age of 12 years had 1.54- fold risk (95% CI: 1.07-2.22) of GBC , compared with those with start menstruating before 12 years or younger, with significant linear trend test (P-*trend*=0.04). No association was observed for number of spontaneous or for induced abortion with GBC risk. No significant association was observed either with oral contraceptive use or duration of usage **[Table 2.24].**

Table 2.4 Selected characteristics of study participants by case -control status

Demographic Information	Category	GBC(n=1,170)		Control(n=2,525)	
		Male(%)	Female(%)	Male(%)	Female(%)
		(N=380)	(N=790)	(N=799)	(N=1726)
Age	20-29	11(2.89%)	11(1.39)	49(6.13)	82(4.75)
	30-39	54(14.2%)	102(12.91)	139(17.39)	410(23.75)
	40-49	93(24.47)	254(32.15)	216(27.03)	599(34.70)
	50-59	123(32.36)	278(35.18)	247(30.91)	453(26.24)
	60+	99(0.26)	145(18.48)	148(18.52)	182(10.54)
	Missing	0	0	0	0
	Mean(±SD)	49.85(10.21)		46.03(10.70)	
Current Residential Region[a]	North	153(40.26)	381(48.22)	230(28.78)	389(22.53)
	North-east	145(38.15)	250(31.64)	229(28.66)	403(23.34)
	Central	25(6.57)	57(7.21)	49(6.13)	105(6.08)
	South	1(0.26)	2(0.25)	5(0.12)	29(1.68)
	West	56(14.73)	100(12.65)	286(35.79)	800(46.34)
	Missing	0	0	0	0
Education	Illiterate	25(0.26)	294(37.21)	20(2.50)	219(12.68)
	Literate	7(1.84)	47(5.94)	27(3.37)	87(5.04)
	Less than 5 years of schooling	27(7.10)	61(7.72)	42(5.25)	109(6.31)
	5-8 years of schooling	90(23.68)	170(21.51)	147(18.39)	417(24.15)
	High School	119(31.31)	138(17.46)	232(29.03)	517(29.95)
	College graduation and more	112(29.47)	75(9.49)	329(41.17)	374(21.66)
	Missing	0	5(0.63)	2(0.25)	3(0.17)
Birth region[b]	High Risk Region	311(81.84)	668(84.55)	469(58.69)	781(45.24)
	Low Risk Region	69(18.15)	120(15.18)	329(41.17)	945(54.75)
	Missing	0	2(0.25)	1(0.12)	0
Gallstone History	No	257(67.63)	436(55.18)	786(98.37)	1669(98.43)
	Yes	120(31.57)	348(20.16)	13(0.12)	47(2.72)
	Missing	3	6	0	10

[a]North (Uttar Pradesh, Bihar, Delhi, Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Chandigarh and Uttarakhand),North-East (Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Tripura, West Bengal, Jharkhand and Orissa), West (Goa, Gujarat, Daman & Diu, Dadra & Nagar Haveli, Maharashtra), South (Andhra Pradesh, Karnataka, Kerala, Lakshadweep, Andaman & Nicobar ,Tamil Nadu, Telangana), and Central (Madhya Pradesh and Chhattisgarh).

[b]High and low risk GBC regions based on incidence data from the National Cancer Registry Programme and Cancer Atlas of India: Bihar, Delhi, Himachal Pradesh, Punjab, Chandigarh, Rajasthan, Uttarakhand, Uttar Pradesh, Assam, Tripura, Sikkim, Jharkhand and West Bengal were classified as high risk regions, the remaining states and territories of India were classified as low risk regions

Table 2.5 Length of residence in high risk region and risk of developing gbc: estimates from case-control study in mumbai (2010-2015)

Parameters	All(Cases: 1,170 , Controls: 2,525)			Men(Cases: 380 , Controls: 799)			Women(Cases: 790, Controls: 1,726)		
	N(Cases| Controls)*	OR (95%CI)†	OR (95%CI)‡	N(Cases Controls)*	OR (95%CI) §	OR (95%CI) ?	N(Cases| Controls)*	OR (95%CI) §	OR (95%CI)'
Birth Place									
Low Risk Region	189|1274	**Reference**		69|329	**Reference**		120|945	**Reference**	
High Risk Region**	979|1250	5.76 (4.77-6.95)	4.82 (3.87-5.99)	311|469	3.62 (2.64-4.96)	3.17 (2.23-4.50)	668|781	7.15 (5.65-9.06)	6.04 (4.52-8.07)
Length of residence in high risk region									
Never lived	169|1,216	**Reference**		66|317	**Reference**		103|899	**Reference**	
Ever lived	995|1302	6.14 (5.04-7.46)	5.09 (4.06-6.38)	313|479	3.59 (2.61-4.94)	3.15 (2.20-4.49)	682|823	8.00 (6.24-10.27)	6.72 (4.95-9.12)
<20 years	46|92	4.01 (2.67-6.01)	3.04 (1.85-4.97)	15|30	2.83 (1.42-5.62)	3.16 (1.51-6.62)	31|62	4.66 (2.81-7.72)	2.49 (1.25-4.95)
≥20 years and less than lifetime	97|171	4.12 (3.02-5.62)	4.02 (2.82-5.73)	39|67	2.86 (1.76-4.64)	2.88 (1.69-4.91)	58|104	5.03 (3.36-7.53)	5.43 (3.33-8.86)
Lifetime	852|1,039	6.85 (5.59-8.38)	5.58 (4.42-7.05)	259|382	3.84 (2.76-5.35)	3.21 (2.21-4.66)	593|657	9.12 (7.05-11.80)	7.65 (5.58-10.49)
$P_{Trend\ test}$		≤0.001	≤0.001		≤0.001	≤0.001		≤0.001	≤0.001
Migration from high to low risk region									
No	1045|2318	**Reference**		328|715	**Reference**		717|1603	**Reference**	
Yes	123|206	1.28 (1.00-1.64)	1.36 (1.02-1.82)	52|83	1.34 (0.92-1.95)	1.63 (1.08-2.46)	71|123	1.27 (0.92-1.76)	1.21 (0.80-1.84)

*Cases and Controls

†Adjusted for age(continuous), gender , education(Less than 5 years of schooling, ≥5 year of education), current residential region

‡Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region, waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and smoking habits(yes/no)

§ Adjusted for variables as † except gender

? Adjusted as ‡ except gender

' Adjusted for variables as ‡ except gender. Number of full term pregnancies(Continuous) also included in the model

** High and low risk GBC regions based on incidence data from the National Cancer Registry Programme and Cancer Atlas of India: Bihar, Delhi, Himachal Pradesh, Punjab, Chandigarh, Rajasthan, Uttarakhand, Uttar Pradesh, Assam, Tripura, Sikkim, Jharkhand and West Bengal were classified as high risk regions, the remaining states and territories of India were classified as low risk regions.

Table 2.6 Odds ratios and 95% confidence intervals for GBC in relation to gallstone and gallstone duration (All study participants)

Gallstone	All(GBC: 1,170 , Controls: 2,525)									
	Self reported gallstone[a]					Self reported gallstone using stringent definition[b]				
	N(Cases| Controls)	OR (95%CI)[c]	P[c]	OR (95%CI)[d]	P[d]	N(Cases | Controls)	OR (95%CI)[c]	P[c]	OR (95%CI)[d]	P[d]
Not present	693|2,455	**Reference**		**Reference**		1,016|2,461	**Reference**		**Reference**	
Present	468|60	29.76 (22.24-39.82)	≤ 0.001	28.94 (21.55-38.86)	≤ 0.001	145|54	6.70 (4.81-9.35)	≤ 0.001	6.10 (4.34-8.56)	≤ 0.001
Missing	9|10					9|10				
Gall stone duration in years (self reported / Self reported gallstone using stringent definition)										
Not present	693|2,455	**Reference**		**Reference**		1,016|2,461	**Reference**		**Reference**	
(≤1 / 1 to 2)	383|9	162.76 (83.15-318.58)	≤ 0.001	156.77 (80.00-307.20)	≤ 0.001	84|11	19.89 (10.43-37.93)	≤ 0.001	16.60 (8.64-31.89)	≤ 0.001
(2 to 4 /3 to 4)	43|16	10.72 (5.88-19.56)	≤ 0.001	9.54 (5.16-17.62)	≤ 0.001	19|8	5.92 (2.52-13.09)	≤ 0.001	5.60 (2.35-13.35)	≤ 0.001
(≥5)	30|35	3.04 (1.81-5.12)	≤ 0.001	3.02 (1.78-5.10)	≤ 0.001	30|35	1.97 (1.18-3.03)	0.009	1.93 (1.15-3.24)	0.013
Missing	21|10					21|10				
P trend			≤ 0.001		≤ 0.001			≤ 0.001		≤ 0.001

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratio

[a]Self reported Gallstone; as per reported by study participant either present/not present. Gallstone duration categories: gallstone not present (reference), ≤1 year, 2-4 years and ≥ 5 years.

[b]Self reported gallstone using stringent definition ; Gallstone history was ascertained using definition of self reported gallstone; however those gallstones diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to the date of interview for control were categorized as "not present". Otherwise was categorized as "present". Gallstone duration categories: gallstone not present (reference), 1-2 years, 3-4 years and ≥ 5 years.

[c]Adjusted for age(continuous), gender and education(less than 5 years schooling , ≥5 year of education), current residential region (north, north-east, central, west, south)

[d] Adjusted for age(continuous),gender, current residential region(north, north-east, central, west, south), education(less than 5 years schooling , ≥ year of education, waist to hip ratio (continuous), tobacco chewing and tobacco smoking (yes/no)

Missing values were excluded from analysis

Table 2.7 Odds ratios and 95% confidence intervals for GBC in relation to gallstone and gallstone duration (Men)

Men(GBC: 380, Controls: 799)										
Gallstone	**Self reported gallstone[a]**					**Self reported gallstone using stringent definition[b]**				
	N(Cases| Controls)	**OR (95%CI)[c]**	**P[c]**	**OR (95%CI)[d]**	**P[d]**	**N(Cases| Controls)**	**OR (95%CI)[c]**	**P[c]**	**OR (95%CI)[d]**	**P[d]**
Not present	257|786	**Reference**		**Reference**		340|790	**Reference**		**Reference**	
Present	120|13	30.45 (16.80-55.22)	≤ 0.001	30.89 (16.62-55.22)	≤ 0.001	37|9	9.95 (4.72-20.95)	≤ 0.001	10.29 (4.67-22.66)	≤ 0.001
Missing	3|0					3|0				
Gall stone duration in years (self reported / Self reported gallstone using stringent definition)										
Not present	257|786	**Reference**		**Reference**		340|790	**Reference**		**Reference**	
(≤1/ 1 to 2)	95|4	79.94 (28.96-220.64)	≤ 0.001	74.84 (27.02-207.25)	≤ 0.001	18|5	9.20 (3.36-25.17)	≤ 0.001	7.85 (2.80-21.99)	≤ 0.001
(2 to 4 /3 to 4)	14| 6	7.58 (2.85-20.15)	≤ 0.001	6.65 (2.44-18.10)	≤ 0.001	8|1	18.53 (2.29-149.90)	0.006	17.19 (2.09- 141.34)	0.008
(≥5)	7|3	6.46 (1.63-25.52)	≤ 0.001	9.79 (1.99-48.01)	≤ 0.001	7|3	4.88 (1.24-19.20)	0.023	7.37 (1.51-36.03)	0.013
Missing	7|0					7|0				
P trend			≤ 0.001		≤ 0.001			≤ 0.001		≤ 0.001

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Self reported Gallstone; as per reported by study participant either present/not present. Gallstone duration categories: gallstone not present (reference), ≤1 year, 2-4 years and ≥ 5 years.

[b]Self reported gallstone using stringent definition ; Gallstone history was ascertained using definition of self reported gallstone; however those gallstones diagnosed within a year prior to the date of diagnosis of gallbladder cancer for cases or within a year prior to the date of interview for control were categorized as "not present". Otherwise was categorized as "present". Gallstone duration categories: gallstone not present (reference), 1-2 years, 3-4 years and ≥ 5 years.

[c]Adjusted for age(continuous), education(less than 5 years schooling , ≥5 year of education), current residential region (north, north-east, central, west, south)

[d]Adjusted for age(continuous), education(less than 5 years schooling , ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), tobacco chewing and tobacco smoking (yes/no)

Missing values were excluded from analysis

Table 2.8 Odds ratios and 95% confidence intervals for GBC in relation to gallstone and gallstone duration (Women)

Women(GBC: 790, Controls: 1,726)										
Gallstone	**Self reported gallstone[a]**					**Self reported gallstone using stringent definition[b]**				
	N(Cases| Controls)	**OR (95%CI)[c]**	**P[c]**	**OR (95%CI)[d]**	**P[d]**	**N(Cases| Controls)**	**OR (95%CI)[c]**	**P[c]**	**OR (95%CI)[d]**	**P[d]**
Not present	436|1,669	**Reference**		**Reference**		676|1,671	**Reference**		**Reference**	
Present	348|47	30.21 (21.54-42.38)	≤ 0.001	29.40 (20.65-41.86)	≤ 0.001	108|45	5.97 (4.09-8.72)	≤ 0.001	5.16 (3.51-7.59)	≤ 0.001
Missing	6|10					6|10				
Gall stone duration in years (self reported / Self reported gallstone using stringent definition)										
Not present	436 | 1,699	**Reference**		**Reference**		676|1,671	**Reference**		**Reference**	
(≤1 / 1 to 2)	288|5	237.24 (96.61-582.56)	≤ 0.001	221.58 (89.91-546.10)	≤ 0.001	66|6	29.25 (12.37-69.14)	≤ 0.001	27.15 (10.49-70.27)	≤ 0.001
(2 to 4 /3 to 4)	29|10	13.12 (6.09-28.23)	≤ 0.001	11.29 (4.90-26.05)	≤ 0.001	11|7	3.89 (1.42-10.63)	0.008	3.41 (1.20-9.69)	0.021
(≥5)	23|32	2.68 (1.50-4.80)	≤ 0.001	2.55 (1.39-4.65)	0.002	23|32	1.66 (0.93-12.37)	0.081	1.53 (0.84-2.78)	0.157
Missing	14|10					14|10				
P trend			≤ 0.001		≤ 0.001			≤ 0.001		≤ 0.001

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Self reported Gallstone; as per reported by study participant either present/not present. Gallstone duration categories for self reported gallstone :gallstone not present (reference), ≤1 year, 2-4 years and ≥ 5 years.

[b]Self reported gallstone using stringent definition; GS was ascertained "not present", if either diagnosed one year prior to date of interview/date of diagnose of GBC or self reports"not present". Categories for "self reported gallstone using stringent definition gallstone not present (reference), 1-2 years, 3-4 years and ≥ 5 years.

[c]Adjusted for age(continuous), education(less than 5 years schooling , ≥5 year of education), current residential region (north, north-east, central, west, south)

[d]Adjusted for age(continuous), education(less than 5 years schooling , ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), tobacco chewing and tobacco smoking (yes/no), and number of full term pregnancies(continuous)

Missing values were excluded from analysis

Table 2.9 Tobacco smoking among study participants

Types of tobacco smoking*	GBC(All=1,170, Men=380, Women=790,)			Controls(All=2525, Men=799, Women=1726,)		
	Men (%)	Women (%)	Total (%)	Men (%)	Women (%)	Total (%)
Bidi smoking[a]	74(19.47)	14(1.77)	88(7.52)	87(10.88)	3(0.17)	90(3.56)
Cigarette smoking	70(18.41)	1(0.12)	71(6.06)	124(15.51)	4(0.2)	128(5.06)
Others(ganja, Dhumati, water pipes /hookah, cigar/cheroots)[b]	14(3.68)	3(0.37)	17(1.45)	3(0.38)	0(0)	3(0.11)

***:** types of tobacco smoking are not mutually exclusive categories

[a]Bidi :thin cigarette filled with tobacco flake and wrapped in a tendu leaf tied with a string at one end.

[b]Water pipes/hookah : single or multi-stemmed, often glass-based instrument for smoking in which the smoke is cooled by water, Cigar : tightly-rolled bundle of dried and fermented tobacco, Cheroot or stogie : cylindrical cigar with both ends clipped during manufacturing, Dhumati : conical cigar made by rolling tobacco leaf in the leaf of another plant.

Table 2.10 Smokeless tobacco use among study participants

Types of smokeless tobacco*	GBC(All=1,170, Men=380, Women=790,)			Controls(All=2,525, Men=799, Women=1,726,)		
	Men (%)	Women (%)	Total (%)	Men (%)	Women (%)	Total (%)
Tobacco *quid*[a]	107(28.15)	77(9.74)	184(15.73)	206(25.78)	72(4.17)	278(11)
Betel leaf quid with Tobacco[b]	58(15.26)	61(7.72)	119(10.17)	95(11.88)	44(2.54)	139(5.50)
Khaini[c]	40(10.52)	13(1.64)	53(4.53)	55(6.88)	14(0.81)	69(2.73)
Mawa[d]	17(4.47)	13(1.64)	30(2.56)	38(4.75)	9(0.52)	52(2.05)
Gutakha[e]	12(3.15)	7(0.88)	19(1.62)	51 (6.38)	12(0.69)	63(2.49)
Masheri[f]	5(1.31)	23(2.91)	28(2.39)	5(0.62)	101(5.85)	106(4.19)
Others(gadakhu,gul,snus, jarda, Pan masala, creamy snuff)[g]	1(0.26)	4(0.50)	5(0.42)	3(0.37)	2(0.11)	5(0.19)

***:** types of tobacco smoking are not mutually exclusive categories

[a] Tobacco *quid* :it is the combination of tobacco, areca nut, slaked lime, and catechu.

[b] Betel leaf *quid* with tobacco: it is the combination of bete leaf, areca nut, slaked lime, and catechu with tobacco.

[c] Khaini: tobacco and slaked lime.

[d] Mawa: mixture of shaving of areca nut, scented tobacco, lime.

[e] Gutakha: dry mixture of crushed areca nut, tobacco, catechu, lime, aromas , flavouring and other additives.

[f] Mishri: roasted, powdered preparation made by baking tobacco on a hot metal plate until it is uniformly black.

[g] Gudhaku: paste made of tobacco and molasses, gul: pyrolysed tobacco product., pan masala: commercial preparation containing areca nut, slaked lime, catechu and condiments with powdered tobacco) , creamy snuff (commercial preparations of tobacco paste marketed in toothpaste-like tubes which are advertised as possessing anti-bacterial activity and being good for the gums and teeth).

Table 2.11 Odds ratios and 95% confidence intervals for GBC in relation to tobacco smoking (All study participants)

Tobacco smoking	(GBC: 1,170, Control: 2,525)				
	N(Cases| Controls)	OR (95%CI)[a]	P[a]	OR (95%CI)[b]	P[b]
Any type of tobacco smoking status[c]					
Non-smoker	1028|2266	**Reference**		**Reference**	
Smoker	141|258	1.08 (0.83-1.40)	0.542	1.08 (0.80-1.45)	0.596
Bidi smoking status					
Non-smoker	1028|2266	**Reference**		**Reference**	
Bidi-smoker	88|90	1.70 (1.21-2.39)	0.002	1.87 (1.28-2.73)	0.001
Bidi smoking/day					
Non-smoker	1028|2266	**Reference**		**Reference**	
1 to 14 bidi/day	48|55	1.47 (0.96-2.25)	0.073	1.59 (0.98-2.57)	0.056
15 to 29 bidi/day	22|20	2.05 (1.07-3.90)	0.029	2.51 (1.23-5.10)	0.011
>29 bidi/day	16|14	1.96 (0.92-4.21)	0.081	1.83 (0.80-4.20)	0.15
P trend			0.003		≤0.001
Bidi Smoking duration(years)					
Non-smoker	1028|2266	**Reference**		**Reference**	
1 to 10 years	15|21	1.36 (0.67-2.73)	0.384	1.30 (0.58-2.90)	0.514
11 to 20 years	29|33	1.73 (1.01-2.96)	0.044	1.96 (1.09-3.52)	0.023
>20 years	44|36	1.86 (1.15-3.02)	0.011	2.13 (1.24-3.65)	0.006
P trend			0.002		≤0.001
Cumulative years of Bidi smoking[d]					
Non-smoker	1028|2266	**Reference**		**Reference**	
1 to 199	37|47	1.37 (0.85-2.18)	0.187	1.45 (0.85-2.45)	0.163
200-400	16|17	1.77 (0.86-3.66)	0.119	2.29 (1.03-5.11)	0.041
>400	35|26	2.27 (1.31-3.92)	0.003	2.41 (1.32-4.39)	0.004
P trend			0.001		≤0.001

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), gender , education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing (yes/no)

[c]Any type of tobacco smoking : included different forms of smoking such as cigarette, bidi(thin cigarette filled with tobacco flake and wrapped in a tendu leaf tied with a sytring at one end),cheroot or stogie(cylindrical cigar with both ends clipped during manufacturing), cigar (tightly rolled bundle of dried and fermented tobacco), water pipes/hookah(single or multi-stemmed,often glass based instrument for smoking in which the smoke is cooled by water),roll your own (cigarette made from loose tobacco and rolling paper), chuttas (coarsely prepared cheroots), dhumti (conical cigar made by rolling tobacco leaf in the leaf of another plant), hooklis (clay pipe) , chillum (straight conical pipe with end-to-end channel,traditionally made of clay).

[d]Number of bidis smoked * duration of smoking

Missing values were excluded from analysis

Table 2.12 Odds ratios and 95% confidence intervals for GBC in relation to any type of smokeless tobacco (All study participants)

Any type of smokeless tobacco	(GBC: 1,170, Control: 2525)				
	N(Cases| Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Any type of smokeless tobacco					
No	875|1985	Reference		Reference	
Yes	295|540	1.04 (0.87-1.25)	0.595	1.15 (0.80-1.45)	0.178
Any type of smokeless tobacco /day					
No	875|1985	Reference		Reference	
1 to 5(times)/day	123|294	0.82 (0.64-1.04)	0.116	0.90 (0.68-1.19)	0.487
>5(times)/day	170|243	1.37 (1.07-1.75)	0.01	1.47 (1.11-1.95)	0.006

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), gender and education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco smoking (yes/no)

Missing values were excluded from analysis

Table 2.13 Odds ratios and 95% confidence intervals for GBC in relation to tobacco quid (All study participants)

Smokeless tobacco	(GBC: 1,170, Control: 2,525)				
	N(Cases| Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Tobacco *quid* chewing					
Non tobacco chewer	875|1985	**Reference**		**Reference**	
chewers	184|278	1.25 (0.99-1.56)	0.051	1.27 (0.97-1.64)	0.072
Tobacco *quid* chewing /day					
Non tobacco chewer	875|1985	**Reference**		**Reference**	
1 to 5(times)/day	65|120	0.95 (0.68-1.34)	0.802	0.87 (0.58-1.29)	0.497
>5(times)/day	116|149	1.52 (1.15-2.00)	0.003	1.60 (1.17-2.20)	0.003
P trend			0.007		0.011
Tobacco *quid* chewing duration (years)					
Non tobacco chewer	875|1985	**Reference**		**Reference**	
1 to 19 years	48|105	0.97 (0.66-1.41)	0.879	0.91 (0.58-1.41)	0.69
>19 years	132|168	1.39 (1.07-1.82)	0.013	1.43 (1.05-1.94)	0.02
P trend			≤0.001		0.036

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), gender and education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco smoking (yes/no).

Tobacco *quid i*s the combination of tobacco, areca nut, slaked lime, and catechu.

Missing values were excluded from analysis

Table 2.14 Odds ratios and 95% confidence intervals for GBC in relation to betel leaf quid with tobacco (All study participants)

Variable	ALL				
	N(Cases| Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Betel leaf *quid* with tobacco					
Non-tobacco chewers	875|1985	**Reference**		**Reference**	
Yes	119|139	1.57 (1.19-2.08)	0.001	1.55 (1.12-2.13)	0.007
Betel leaf *quid* with tobacco/day					
Non-tobacco chewers	875|1985	**Reference**		**Reference**	
1 to 5(times)/day	86|111	1.39 (1.01-1.90)	0.04	1.31 (0.91-1.89)	0.14
>5(times)/day	30|25	2.44 (1.39-4.26)	0.001	2.61 (1.40-4.88)	0.003
P trend			≤0.001		0.002
Betel leaf *quid* with tobacco(year)					
Non-tobacco chewers	875|1985	**Reference**		**Reference**	
1 to 19 years	36|54	1.59 (1.01-2.49)	0.044	1.58 (0.94--2.65)	0.081
>19 years	82|80	1.64 (1.16-2.31)	0.004	1.60 (1.08-2.37)	0.019
P trend			0.001		0.007

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), gender and education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco smoking (yes/no)

Betel leaf *quid with* tobacco is the combination of betel leaf, areca nut, slaked lime, and catechu with tobacco.

Missing values were excluded from analysis

Table 2.15 Odds ratios and 95% confidence intervals for GBC in relation to general and abdominal obesity (Men)

Obesity	Categories	Men(GBC: 380, Control: 799)				
		Cases| Controls	OR[a] (95% CI)	P[a]	OR[b] (95%CI)	P[b]
BMI (Kg/m2)-world	<18.5	57|50	2.10 (1.38-3.20)	≤0.001	2.81 (1.74-4.53)	≤0.001
	18.5-24.9	230|420	**Reference**		**Reference**	
	25.0-29.9	65|253	0.44 (0.32-0.61)	≤0.001	0.35 (0.24-0.52)	≤0.001
	≥30	3 | 64	0.08 (0.02-0.27)	≤0.001	0.08 (0.02-0.52)	≤0.001
	Missing	25 | 12				
	P trend			≤0.001		≤0.001
Height (in cm)	≤160	132|250	**Reference**		**Reference**	
	161-164	92|183	0.98 (0.70-1.37)	0.93	1.05 (0.72-1.51)	0.792
	165-168	64|174	0.72 (0.50-1.04)	0.083	0.68 (0.45-1.03)	0.072
	≥169	70|182	0.78 (0.55-1.12)	0.185	0.63 (0.41-0.96)	0.033
	Missing	22|10				
	P trend			0.077		0.011
Weight (in Kg)	≤53	128|111	**Reference**		**Reference**	
	54-62	115|222	0.43 (0.30-0.61)	≤0.001	0.31 (0.21-0.47)	≤0.001
	>62	120|454	0.21 (0.15-0.30)	≤0.001	0.12 (0.083-0.19)	≤0.001
	Missing	17|12				
	P trend			≤0.001		≤0.001
Waist circumference (in cm)[c]	≤83	152|227	**Reference**		**Reference**	
	84-92	93|227	0.60 (0.44-0.83)	0.002	1.39 (0.89-2.17)	0.088
	≥93	65|192	0.46 (0.32-0.66)	≤0.001	2.84 (1.51-5.34)	0.001
	Missing	70|153				
	P trend			≤0.001		0.002
Hip circumference (in cm)[c]	≤90	196|279	**Reference**		**Reference**	
	91-99	132|327	0.56 (0.42-0.74)	≤0.001	0.92 (0.62-1.36)	0.68
	≥100	32|180	0.24 (0.15-0.36)	≤0.001	0.84 (0.41-1.52)	0.584
	Missing	20|13				
	P trend			≤0.001		0.568

continue..

Obesity	Categories	Men(GBC: 380, Control: 799)				
		Cases| Controls	OR[a] (95% CI)	P[a]	OR[b] (95%CI)	P[b]
Waist-to-hip ratio[c]	≤0.90	136|264	Reference		Reference	
	0.91-0.96	84|211	0.77 (0.55-1.07)	0.128	1.24 (0.83-1.87)	0.284
	≥0.97	139|311	0.78 (0.58-1.06)	0.119	1.93 (1.30-2.86)	0.001
	Missing	21|13				
	P trend			0.123		0.001
Body size at age 10 (using pictogram)[c]	< 3	103|334	Reference		Reference	
	3 to 4	157|273	1.88 (1.39-2.53)	≤0.001	1.83 (1.28-2.63)	0.001
	≥ 5	118|178	2.04 (1.47-2.82)	≤0.001	2.00 (1.35-2.96)	≤0.001
	Missing	2|14				
	P trend			≤0.001		≤0.001
Body size at age 20 (using pictogram)[c]	< 3	47|160	Reference		Reference	
	3 to 4	169|360	1.54 (1.06-2.25)	0.023	1.64 (1.04-2.59)	0.032
	≥ 5	161|267	1.87 (1.28-2.75)	0.001	2.3 (1.45-3.69)	≤0.001
	Missing	3|12	1.33 (1.11-1.59)	0.002	1.49 (1.20-1.85)	≤0.001
	P trend					
Body size at current age (using pictogram)[c]	< 3	80|80	Reference		Reference	
	3 to 4	158|248	0.63 (0.43-0.93)	0.02	0.91 (0.58-1.43)	0.709
	≥ 5	140|460	0.28 (0.19-0.41)	≤0.001	0.65 (0.39-1.07)	0.091
	Missing	2|11				
	P trend			≤0.001		0.053

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age, gender and education(less than 5 years schooling , ≥ year of education),current residential region(north, north-east, central, west, south)

[b]Adjusted for age, education(less than 5 years schooling , ≥ year of education), current residential region(north, north-east, central, west, south), gallstone (present/not present)waist to hip ratio (continuous), tobacco chewing and tobacco smoking (yes/no)

[c]Same as b. However, adjusted for BMI (continuous) instead of waist to hip ratio

Missing values were excluded from analysis

Table 2.16 Odds ratios and 95% confidence intervals for GBC in relation to general and abdominal obesity (Women)

Variable	Categories	Women(GBC: 790, Control: 1,726)				
		Cases| Controls	OR[a] (95% CI)	P[a]	OR[b] (95%CI)	P[b]
BMI (Kg/m2)-world	< 18.5	132|93	2.55 (1.86-3.49)	≤0.001	3.43 (2.35-5.01)	≤0.001
	18.5-24.9	402|785	Reference		Reference	
	25.0-29.9	173|585	0.59 (0.47-0.73)	≤0.001	0.43 (0.32-0.57)	≤0.001
	≥ 30	38|251	0.29 (0.20-0.43)	≤0.001	0.22 (0.14-0.36)	≤0.001
	Missing	45|12				
	P trend			≤0.001		≤0.001
Height (in cm)	≤ 150	397|752	Reference		Reference	
	151-155	222|505	0.89 (0.72-1.09)	0.28	1.01 (0.77-1.31)	0.937
	156-160	100|345	0.62 (0.48-0.82)	0.001	0.64 (0.45-0.89)	0.01
	≥ 161	29|112	0.58 (0.37-0.91)	0.019	0.71 (0.42-1.21)	0.215
	Missing	42|12				
	P trend			≤0.001		0.018
Weight (in Kg)	≤ 60	639|1,065	Reference		Reference	
	61-65	57|258	0.40 (0.29-0.55)	≤0.001	0.36 (0.24-0.54)	≤0.001
	> 65	64|394	0.29 (0.22-0.40)	≤0.001	0.25 (0.17-0.36)	≤0.001
	Missing	30|9				
	P trend			≤0.001		≤0.001
Waist circumference (in cm)c	≤ 79	340|705	Reference		Reference	
	80-85	126|316	0.87 (0.67-1.13)	0.31	1.53 (1.07-2.18)	0.017
	≥ 86	208|605	0.69 (0.55-0.86)	0.001	2.14 (1.46-3.14)	≤0.001
	Missing	116|100				
	P trend			0.001		≤0.001
Hip circumference (in cm)c	≤ 90	370|448	Reference		Reference	
	91-99	241|610	0.48 (0.38-0.59)	≤0.001	0.59 (0.44-0.81)	0.001
	≥ 100	150|656	0.27 (0.21-0.30)	≤0.001	0.49 (0.31-0.77)	0.002
	Missing	29|12				
	P trend			≤0.001		0.001

continue..

Variable	Categories	Women(GBC: 790, Control: 1,726)				
		Cases| Controls	OR[a] (95% CI)	P[a]	OR[b] (95%CI)	P[b]
Waist-to-hip ratio[c]	≤ 0.84	265|952	Reference		Reference	
	0.85-0.94	366|618	1.90 (1.56-2.32)	≤0.001	2.49 (1.90-3.25)	≤0.001
	≥ 0.95	128|144	2.67 (1.99-3.59)	≤0.001	4.68 (3.20-6.86)	≤0.001
	Missing	31|12				
	P trend			≤0.001		≤0.001
Body size at age 10 (using pictogram)[c]	< 3	235|774	Reference		Reference	
	3 to 4	232|507	1.63 (1.30-2.05)	≤0.001	1.64 (1.22-2.20)	0.001
	≥ 5	298|426	2.25 (1.80-2.80)	≤0.001	2.52 (1.90-3.34)	≤0.001
	Missing	25|19				
	P trend			≤0.001		≤0.001
Body size at age 20 (using pictogram)[c]	< 3	91|405	Reference		Reference	
	3 to 4	298|689	2.07 (1.57-2.73)	≤0.001	1.91 (1.33-2.74)	≤0.001
	≥5	376|616	2.65 (2.01-3.48)	≤0.001	2.98 (2.10-4.23)	≤0.001
	Missing	25|16				
	P trend			≤0.001		≤0.001
Body size at current age (using pictogram)[c]	< 3	181|122	Reference		Reference	
	3 to 4	274|491	0.43 (0.32-0.58)	≤0.001	0.67 (0.46-0.96)	0.031
	≥ 5	311|1,099	0.21 (0.16-0.28)	≤0.001	0.50 (0.33-0.74)	0.001
	Missing	24|14				
	P trend			≤0.001		0.001

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), education(less than 5 years schooling , ≥ year of education),current residential region(north, north-east, central, west, south)

[b]Adjusted for age, education(less than 5 years schooling , ≥ year of education), current residential region(north, north-east, central, west, south), gallstone (present/not present)waist to hip ratio (continuous), tobacco chewing and tobacco smoking (yes/no), number of full term pregnancies(continuous)

[c]Same as b. However, adjusted for BMI (continuous) instead of waist to hip ratio

Missing values were excluded from analysis

Table 2.17 Adjusted odds ratios and 95 % confidence intervals for GBC in relation to eating habits (All study participants)

Eating Habits	All(Case: 1,170 , Control: 2,525)				
	N(Cases | Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Lifelong vegetarianism	225|522	Reference		Reference	
Non-vegetarianism	834|1,802	1.22(1.01-1.48)	0.035	1.02(0.81-1.27)	0.848
<30 years of Non-vegetarianism	30|152	0.94(0.59-1.50)	0.825	0.96(0.56-1.63)	0.885
≥ 30 years of Non-vegetarianism	800|1635	1.23(1.02-1.49)	0.027	1.02(0.81-1.27)	0.839
P trend			0.019		0.813

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

Vegetarian: Study participants following practice of plant-based diet without the inclusion of eggs and meat one year before to the date of interview for controls and before the diagnosis of cancer for cases were categorized as "vegetarian".

Non-vegetarian: Study participants following practice of any meat consumption were categorized as "non-vegetarian".

Lifelong vegetarians: are those practiced vegetarian diet over their entire course of life (one year prior to interview for controls and before the diagnosis of cancer for cases).

[a]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking (yes/no)

Missing values are excluded from analysis

Table 2.18 Odds ratios and 95 % confidence intervals for GBC in relation to dietary variables and spices (All study participants)

Dietary Variables	All study participants (Cases:1,170 , Controls: 2,525)				
	N(Cases | Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Any fruit*					
Lowest quartile	387|617	**Reference**		**Reference**	
2nd quartile	301|643	0.76 (0.63-0.93)	0.008	0.61 (0.49-0.77)	≤0.001
3rd quartile	238|619	0.69 (0.56-0.85)	≤0.001	0.58 (0.46-0.74)	≤0.001
Highest quartile	215|612	0.69 (0.55-0.85)	0.001	0.53 (0.41-0.69)	≤0.001
Missing	29|34				
P trend			≤0.001		≤0.001
Citrus fruits**					
Lowest quartile	342|549	**Reference**		**Reference**	
2nd quartile	319|661	0.81 (0.67-1.00)	0.051	0.76 (0.60-0.96)	0.026
3rd quartile	127|443	0.49 (0.38-0.63)	≤0.001	0.45 (0.33-0.60)	≤0.001
Highest quartile	181|436	0.81 (0.64-1.02)	0.087	0.63 (0.47-0.83)	0.001
Missing	201|436				
P trend			0.001		≤0.001
Non-citrus fruits***					
Lowest quartile	406|639	**Reference**		**Reference**	
2nd quartile	287|633	0.75 (0.61-0.91)	0.004	0.64 (0.51-0.81)	≤0.001
3rd quartile	234|595	0.69 (0.56-0.85)	0.001	0.59 (0.46-0.75)	≤0.001
Highest quartile	214|622	0.66 (0.54-0.82)	≤0.001	0.55 (0.42-0.70)	≤0.001
Missing	29|36				
P trend			≤0.001		≤0.001
Any vegetable[†]					
Lowest quartile	427|645	**Reference**		**Reference**	
2nd quartile	240|619	0.59 (0.48-0.72)	≤0.001	0.52 (0.41-0.66)	≤0.001
3rd quartile	243|628	0.65 (0.53-0.80)	≤0.001	0.59 (0.46-0.74)	≤0.001
Highest quartile	250|627	0.75 (0.62-0.92)	0.007	0.63 (0.49-0.80)	≤0.001
Missing	10|6				
P trend			0.005		≤0.001

continue…

Dietary Variables	All study participants (Cases:1,170 , Controls: 2,525)				
	N(Cases | Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Leafy green vegetables[††]					
Lowest quartile	443|715	**Reference**		**Reference**	
2nd quartile	267|637	0.67 (0.55-0.81)	≤0.001	0.70 (0.56-0.88)	0.002
3rd quartile	217|560	0.63 (0.51-0.78)	≤0.001	0.66 (0.52-0.84)	0.001
Highest quartile	220|571	0.68 (0.55-0.83)	≤0.001	0.66 (0.52-0.85)	0.001
Missing	23|42				
P trend			≤0.001		≤0.001
Root vegetables[†††]					
Lowest quartile	368|631	**Reference**		**Reference**	
2nd quartile	291|633	0.84 (0.69-1.03)	0.096	0.76 (0.60-0.95)	0.019
3rd quartile	241|623	0.75 (0.61-0.92)	0.007	0.67 (0.53-0.86)	0.002
Highest quartile	257|627	0.90 (0.73-1.11)	0.346	0.75 (0.59-0.96)	0.022
Missing	13|11				
P trend			0.17		0.008
Fruiting vegetables[?]					
Lowest quartile	433|636	**Reference**		**Reference**	
2nd quartile	254|654	0.57 (0.47-0.70)	≤0.001	0.52 (0.42-0.66)	≤0.001
3rd quartile	214|603	0.58 (0.47-0.71)	≤0.001	0.49 (0.38-0.63)	≤0.001
Highest quartile	259|625	0.74 (0.60-0.90)	0.003	0.60 (0.47-0.76)	≤0.001
Missing	10|7				
P trend			0.001		≤0.001
Cruciferous vegetables[??]					
Lowest quartile	406|729	**Reference**		**Reference**	
2nd quartile	445|1066	0.74 (0.62-0.88)	0.001	0.69 (0.56-0.84)	≤0.001
3rd quartile	71|199	0.65 (0.47-0.88)	0.007	0.56 (0.39-0.81)	0.002
Highest quartile	229|502	0.88 (0.72-1.09)	0.267	0.68 (0.53-0.87)	0.003
Missing	19|29				
P trend			0.196		0.001

continue…

Dietary Variables	All study participants (Cases:1,170 , Controls: 2,525)				
	N(Cases | Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Tomatoes					
Lowest quartile	368|634	**Reference**		**Reference**	
2nd quartile	275|674	0.71 (0.58-0.87)	0.001	0.63 (0.50-0.79)	≤0.001
3rd quartile	179|536	0.64 (0.51-0.79)	≤0.001	0.52 (0.40-0.67)	≤0.001
Highest quartile	226|484	0.93 (0.75-1.15)	0.542	0.74 (0.57-0.95)	0.021
Missing	122|197				
P trend		0.95(0.89-1.02)	0.181	0.87(0.80-0.94)	0.001
Onion and Garlic					
Lowest quartile	360|617	**Reference**		**Reference**	
2nd quartile	295|666	0.76 (0.62-0.92)	0.007	0.62 (0.49-0.78)	≤0.001
3rd quartile	168|489	0.64 (0.51-0.81)	≤0.001	0.49 (0.37-0.64)	≤0.001
Highest quartile	243|590	0.81 (0.66-1.00)	0.056	0.67 (0.52-0.85)	0.001
Missing	104|163				
P trend			0.019		≤0.001
Any Cereal[c???]					
Lowest quartile	332|638	**Reference**		**Reference**	
2nd quartile	315|667	0.93 (0.76-1.13)	0.512	0.80 (0.63-1.01)	0.068
3rd quartile	304|596	0.98 (0.80-1.19)	0.848	1.00 (0.79-1.27)	0.952
Highest quartile	211|616	0.70 (0.57-0.87)	0.002	0.69 (0.54-0.89)	0.005
Missing	8|8				
P trend			0.006		0.046
Any Pulses[c#]					
Lowest quartile	340|660	**Reference**		**Reference**	
2nd quartile	247|616	0.84 (0.68-1.03)	0.101	0.82 (0.64-1.06)	0.136
3rd quartile	274|625	0.90 (0.73-1.10)	0.322	0.98 (0.77-1.25)	0.903
Highest quartile	298|617	1.02 (0.84-1.25)	0.79	1.18 (0.94-1.50)	0.147
Missing	11|7				
P trend			0.714		0.087

continue…

Dietary Variables	All study participants (Cases:1,170 , Controls: 2,525)				
	N(Cases | Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Any Spice [£c]					
Lowest quartile	364|621	**Reference**		**Reference**	
2nd quartile	243|635	0.67 (0.55-0.83)	<0.001	0.68 (0.53-0.88)	0.003
3rd quartile	271|604	0.77 (0.62-0.94)	0.011	0.80 (0.63-1.01)	0.069
Highest quartile	271|619	0.74 (0.61-0.91)	0.005	0.88 (0.69-1.11)	0.289
Missing	21|46				
P trend			0.014		0.444
Fenugreek[c]					
Lowest quartile	324|844	**Reference**		**Reference**	
2nd quartile	488|232	1.33(1.07-1.64)	0.008	1.42(1.1-1.8)	0.006
3rd quartile	324|697	1.29(1.06-1.56)	0.009	1.33(1.05-1.68)	0.016
Highest quartile	275|482	1.53(1.24-1.87)	≤0.001	1.91(1.5-2.43)	≤0.001
Missing	15|14				
P trend			≤0.001		≤0.001

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), gender, waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking (yes/no) , any meat consumption

[c]not adjusted for any meat consumption

[*]Any fruit included: Banana, Apple, Mango, Melon, Watermelon, Pear, Grape, Citrus fruit (Orange/ Sweet lime)

[**]Citrus fruits included: Orange & Sweet lime

[***]non citrus food included: Banana, Apple, Mango, Melon, Watermelon, Pear, Grape

[†]Any vegetable included: raw vegetables (Cucumber, Carrots, Radish, Onions, Garlic, Tomatoes, Beet root) & Cooked vegetables (Potatoes, Onions, Garlic, Tomatoes, Ladies fingers , Brinjal /Egg plant, Bitter Gourd, Carrot, Radish, Turnip, Beetroot, Cauliflower, Cabbage, Pumpkin, Spinach, Colocasia, Bottle gourd, Beans, Ivy Gourd, Fenugreek Leaves, Drumstick, Cow Pea leaves, Mixed vegetables)

[††]Leafy green vegetables included: Spinach, Fenugreek Leaves, Cowpea leaves

[†††]Root vegetables included : Raw vegetables (Carrots, Radish, Onions, Garlic, Beet root) & Cooked vegetables (Potatoes, Onions, Garlic, Carrot, Radish, Turnip, Beetroot, Colocasia)

[?]Fruiting vegetables included: Raw Vegetables (Cucumber, Tomatoes & Cooked vegetables: Tomatoes, Ladies fingers , Brinjal /Egg plant, Bitter Gourd, Pumpkin, Bottle gourd, Beans, Ivy Gourd, Drumstick)

[??]Cruciferous vegetables included: Cauliflower, Cabbage

[???] [c]Any Cereal included: Rice, Maize, Bajra , Jowar , Flaked rice , Bread, Chapatti

[#c]Any Pulses included: Lentils, green gram, Bengal gram, Black gram, Red gram, Soya, Moth beans

[£c]Any Spice: Green chillies, Red chillies , Turmeric , Garam Masala which is a blend of ground spices (whole / powder / grind form/ raw /cooked) , Pickles (Any pickle including of prawn pickle)

Missing values were excluded from analysis

Table 2.19 Odds ratios and 95% confidence intervals for GBC in relation meat intake (All study participants)

Dietary variables	All study participants (Cases: 1,170 , Controls: 2,525)				
	N(Cases| Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Any meat*					
Lowest quartile	335|737	**Reference**		**Reference**	
2nd quartile	272|556	1.15 (0.93-1.41)	0.177	1.05 (0.82-1.34)	0.681
3rd quartile	269|597	1.10 (0.89-1.35)	0.355	0.88 (0.69-1.13)	0.332
Highest quartile	281|628	1.23 (1.00-1.51)	0.043	1.07 (0.83-1.37)	0.584
Missing	13|7				
P trend			0.069		0.948
Mutton					
Lowest quartile	481|1,052	**Reference**		**Reference**	
2nd quartile	81|190	1.03 (0.77-1.39)	0.806	1.09 (0.77-1.53)	0.617
3rd quartile	338|726	1.06 (0.88-1.26)	0.501	0.84 (0.67-1.04)	0.113
Highest quartile	247|538	1.04 (0.86-1.27)	0.637	0.94 (0.74-1.19)	0.628
Missing	23|19				
P trend			0.533		0.288
Chicken					
Lowest quartile	439|932	**Reference**		**Reference**	
2nd quartile	178|433	0.92 (0.74-1.15)	0.508	0.83 (0.64-1.08)	0.174
3rd quartile	343|781	1.03 (0.86-1.23)	0.725	0.88 (0.70-1.09)	0.255
Highest quartile	187|364	1.21 (0.97-1.52)	0.081	1.24 (0.95-1.61)	0.111
Missing	23|15				
P trend			0.123		0.44
Fresh Fish					
Lowest quartile	384|925	**Reference**		**Reference**	
2nd quartile	167|372	1.09 (0.87-1.37)	0.442	0.86 (0.65-1.14)	0.307
3rd quartile	320|682	1.20 (0.99-1.45)	0.052	1.10 (0.88-1.38)	0.381
Highest quartile	278|530	1.65 (1.35-2.03)	≤0.001	1.41 (1.10-1.80)	0.006
Missing	21|16				
P trend			≤0.001		0.007

continue…

Dietary variables	All study participants (Cases: 1,170 , Controls: 2,525)				
	N(Cases| Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Dry fish					
Lowest quartile	914|1,828	**Reference**		**Reference**	
Highest quartile	236|676	0.72 (0.60-0.86)	<0.001	0.75 (0.61-0.92)	0.007
Missing	20|21				

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), gender , current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking (yes/no) , all fruit consumption

[*]Any meat: Mutton, Beef, Chicken, Fresh fish, Dry fish, Egg

Missing values were excluded from analysis

Table 2.20 Odds ratios and 95% confidence intervals for GBC in relation to dairy products (All study participants)

Dietary Variables	All(Case: 1,170 , Control: 2,525)				
	N(Cases| Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Any dairy*					
Lowest quartile	322|649	**Reference**		**Reference**	
2nd quartile	259|620	0.92 (0.75-1.13)	0.466	0.91 (0.71-1.16)	0.459
3rd quartile	330|627	1.24 (1.02-1.52)	0.029	1.16 (0.91-1.47)	0.209
Highest quartile	246|623	0.96 (0.78-1.18)	0.729	1.01 (0.78-1.29)	0.925
Missing	13|6				
P trend			0.519		0.474

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

*Any dairy :Milk, Butter, Cheese, Buttermilk, Curd/yogurt, Cottage Cheese, Condensed Milk

[a]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and smoking habits(yes/no)

[b]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking (yes/no)

Missing values were excluded from analysis

Table 2.21 Percentage usage of cooking oil among study

Cooking Oil*	All	Cases (%)	Controls (%)
Mustard Oil	2078(56.23)	914(78.11)	1164(46.09)
Sunflower Oil	1157(31.31)	241(20.59)	916(36.27)
Animal Ghee	1471(39.81)	388(33.16)	1083(42.89)
Soya Oil	562(15.20)	156(13.33)	406(16.07)
Groundnut Oil	547(14.80)	97(8.29)	450(17.82)
Vegetable ghee	388(10.50)	111(9.48)	277(10.97)
Palm Oil	113(3.05)	18(1.53)	95(3.76)

*cooking oil intake is not mutually exclusive

Table 2.22 : Odds ratios and 95% confidence intervals for GBC in relation to cooking oil (All study participants)

Cooking medium	All(Case: 1,170 , Control: 2,525)				
	N(Cases | Controls)	OR(95%CI)[a]	P[a]	OR(95%CI)[b]	P[b]
Sunflower oil					
No	933|1,624	**Reference**		**Reference**	
Yes	237|901	0.49 (0.41-0.58)	≤0.001	0.43 (0.35-0.54)	≤0.001
Lowest tertiles	933|1,624	**Reference**		**Reference**	
2nd tertiles	152|439	0.64 (0.52-0.79)	≤0.001	0.53 (0.40-0.69)	≤0.001
Highest tertiles	85|462	0.34 (0.26-0.44)	≤0.001	0.34 (0.25-0.46)	≤0.001
P trend			≤0.001		≤0.001
Soya Oil					
No	1,019|2,130	**Reference**		**Reference**	
Yes	151|395	0.85 (0.69-1.05)	0.153	1.12 (0.88-1.42)	0.327
Lowest tertiles	1019|2130	**Reference**		**Reference**	
2nd tertiles	124|240	1.15 (0.90-1.45)	0.249	1.47 (1.13-1.92)	0.004
Highest tertiles	27|155	0.39 (0.25-0.60)	≤0.001	0.53 (0.33-0.86)	0.01
P trend			0.004		0.622
Mustard oil					
No	274|1,384	**Reference**		**Reference**	
Yes	896|1,141	4.25 (3.59-5.03)	≤0.001	3.59 (2.96-4.37)	≤0.001
Lowest tertiles	274|1,384	**Reference**		**Reference**	
2nd tertiles	458|556	4.33 (3.57-5.25)	≤0.001	3.80 (3.04-4.75)	≤0.001
Highest tertiles	438|585	4.18 (3.46-5.05)	≤0.001	3.41 (2.73-4.25)	≤0.001
P trend			≤0.001		≤0.001
Animal ghee					
No	793|1,465	**Reference**		**Reference**	
Yes	377|1,060	0.71 (0.60-0.82)	≤0.001	0.76 (0.63-0.91)	0.004
Lowest tertiles	793|1,465	**Reference**		**Reference**	
2nd tertiles	258|701	0.73 (0.61-0.87)	0.001	0.81 (0.66-1.00)	0.052
Highest tertiles	119|359	0.65 (0.51-0.82)	≤0.001	0.66 (0.50-0.88)	0.005
P trend			≤0.001		0.002

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and smoking habits(yes/no)

[b]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking (yes/no) Missing values were excluded from analysis Tertiles categories for cooking oil intake are not mutually exclusive

Table 2.23 Odds ratios and 95% confidence intervals for gallbladder cancer in relation to fenugreek ,fresh fish and mustard oil consumption: Stratified by birth region(All study participants)

Dietary variables	High risk region (Cases : 979 , Control : 1,250)					Low risk region (Cases : 189 , Control :1274)				
	Cases| Controls	OR (95%CI)[a]	P[a]	OR (95%CI)[b]	P[b]	Cases| Controls	OR (95%CI)[a]	P[a]	OR (95%CI)[b]	P[b]
Fenugreek										
Lowest quartile	250|292	**Reference**		**Reference**		**74|552**	**Reference**		**Reference**	
2nd quartile	204|226	1.01 (0.77-1.32)	0.922	1.13 (0.82-1.55)	0.427	28|262	0.77 (0.47-1.26)	0.313	0.82 (0.46-1.47)	0.517
3rd quartile	269|408	0.73 (0.57-0.94)	0.015	0.81 (0.61-1.09)	0.174	55|288	1.40 (0.93-2.10)	0.099	1.35 (0.83-2.21)	0.215
Highest quartile	245|316	0.86 (0.67-1.11)	0.26	1.18 (0.88-1.57)	0.252	30|166	1.23 (0.76-2.00)	0.391	1.25 (0.69-2.26)	0.446
Missing	11|8					2|6				
P Trend			0.067		0.636			0.122		0.218
Fresh Fish										
Lowest quartile	315|441	**Reference**		**Reference**		69|483	**Reference**		**Reference**	
2nd quartile	137|168	1.26 (0.95-1.69)	0.102	1.08 (0.78-1.50)	0.629	30|204	1.13 (0.69-1.85)	0.607	0.78 (0.42-1.45)	0.44
3rd quartile	261|303	1.42 (1.12-1.81)	0.004	1.37 (1.05-1.81)	0.021	59|379	1.29 (0.86-1.94)	0.208	1.20 (0.75-1.93)	0.439
Highest quartile	252|330	1.38 (1.07-1.79)	0.013	1.22 (0.90-1.64)	0.187	26|200	1.19 (0.72-1.99)	0.483	1.00 (0.53-1.89)	0.98
Missing	14|8					5|8				
P Trend			0.004		0.065			0.272		0.584

continue…

Dietary variables	High risk region (Cases : 979 , Control : 1,250)					Low risk region (Cases : 189 , Control :1274)				
	OR (95%CI)[a]	P[a]	OR (95%CI)[b]	P[b]	Cases| Controls	OR (95%CI)[a]	P[a]	OR (95%CI)[b]	P[b]	
Mustard Oil										
No		**Reference**		**Reference**			**Reference**		**Reference**	
Yes		1.33 (10.5-1.68)	0.015	1.35 (1.03-1.77)	0.028		4.78 (3.27-6.99)	≤0.001	1.33 (1.05-1.68)	0.015
Lowest quartile	159|255	**Reference**		**Reference**		113|1,128	**Reference**		**Reference**	
2nd quartile	415|488	1.33 (1.03-1.71)	0.027	1.4 (1.04-1.88)	0.023	43|68	5.69 (3.53-9.16)	≤0.001	3.42 (1.88-6.21)	≤0.001
Highest quartile	405|507	1.33 (1.04-1.71)	0.023	1.30 (0.97-1.74)	0.073	33|78	3.99 (2.44-6.52)	≤0.001	2.46 (1.35-4.48)	0.003
P Trend			0.049		0.176			≤0.001		≤0.001

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south),

[b]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking (yes/no)

** High and low risk GBC regions based on incidence data from the National Cancer Registry Programme and Cancer Atlas of India: Bihar, Delhi, Himachal Pradesh, Punjab, Chandigarh, Rajasthan, Uttarakhand, Uttar Pradesh, Assam, Tripura, Sikkim, Jharkhand and West Bengal were classified as high risk regions, the remaining states and territories of India were classified as low risk regions.

Missing values were excluded from analysis

Table 2.24 Odds ratios and 95% confidence intervals for gallbladder cancer in relation to reproductive factors among female study participants

Reproductive factors	Cases|Controls	OR[a] (95% CI)	P[a]	OR[b] (95%CI)	P[b]
Number of first full term pregnancies					
1	45 | 199	**Reference**		**Reference**	
2	122 | 554	0.88 (0.59-1.29)	0.519	0.93 (0.58-1.50)	0.783
3	177 | 445	1.27 (0.86-1.86)	0.215	1.28 (0.79-2.05)	0.304
≥4	429 | 425	2.34 (1.59-3.43)	≤0.001	2.34 (1.46-3.74)	≤0.001
Missing	0 | 18				
P trend			≤0.001		≤0.001
Risk per increase in number of full-term pregnancy		1.33 (1.25-1.42)	≤0.001	1.31 (1.22-1.41)	≤0.001
Age at full term pregnancy (in years)					
<20	291 | 531	**Reference**		**Reference**	
20-21	198 | 346	1.09 (0.85-1.38)	0.471	1.25 (0.93-1.68)	0.132
22-23	115 | 280	0.90 (0.68-1.19)	0.493	1.00 (0.70-1.42)	0.987
24-25	84 | 182	1.03 (0.75-1.41)	0.823	1.46 (0.98-2.16)	0.059
≥26	82 | 282	0.72 (0.53-0.97)	0.035	1.00 (0.67-1.49)	0.978
Missing	3 | 20				
P trend			0.07		0.542
Total number of pregancies[c]					
0 (Never)	12 | 70	**Reference**		**Reference**	
Ever	773 | 1641	1.47 (0.77-2.80)	0.238	1.04 (0.20-5.44)	0.959
Missing	5 | 15				
Age at menarche(years)					
≤12	86 | 273	**Reference**		**Reference**	
13-14	403 | 926	1.30 (0.98-1.73)	0.062	1.54 (1.07-2.22)	0.018
15-20	262 | 506	1.39 (1.03-1.87)	0.031	1.61 (1.09-2.36)	0.015
Missing	39 | 21				
P trend			0.052		0.04
Risk per year increase in age at menarche		1.02 (1.00-1.04)	0.03	1.01 (0.99-1.03)	0.084

continue…

Reproductive factors	Cases|Controls	OR[a] (95% CI)	P[a]	OR[b] (95%CI)	P[b]
Age at menopause(years)					
≤46	310 | 421	**Reference**		**Reference**	
47-48	88 | 135	0.99 (0.71-1.38)	0.981	1.17 (0.79-1.73)	0.423
49-52	91 | 158	0.70 (0.50-0.98)	0.042	0.81 (0.55-1.21)	0.324
≥53	38 | 61	0.68 (0.42-1.11)	0.128	0.64 (0.35-1.18)	0.158
Missing	15 | 6				
P trend			0.032		0.171
Duration since last birth (in years)[c]					
≤10	133| 391	**Reference**		**Reference**	
11 to 20	267| 582	0.69 (0.51-0.93)	0.016	0.77 (0.53-1.11)	0.163
21 to 30	258| 446	0.47 (0.31-0.70)	≤0.001	0.67 (0.40-1.10)	0.114
>30	110| 193	0.23 (0.13-0.40)	≤0.001	0.44 (0.22-0.87)	0.02
Missing	22|114				
P trend			≤0.001		0.029
Risk per increase in year		0.97 (0.96-0.99)	≤0.001	0.98 (0.97-1.00)	0.093
Number of induced abortions					
0	653 | 1,252	**Reference**		**Reference**	
1	86 | 276	0.87 (0.66-1.14)	0.33	0.90 (0.64-1.27)	0.562
≥2	34 | 113	0.80 (0.53-1.21)	0.311	0.66 (0.39-1.13)	0.136
Missing					
P trend			0.187		0.137
Number of spontaneous abortion					
0	630 | 1,316	**Reference**		**Reference**	
1	102 | 210	1.01 (0.77-1.33)	0.906	0.94 (0.67-1.31)	0.729
≥2	41 | 115	0.77 (0.52-1.14)	0.198	0.78 (0.49-1.25)	0.319
Missing	0 | 0				
P trend			0.324		0.324

continue…

Reproductive factors	Cases|Controls	OR[a] (95% CI)	P[a]	OR[b] (95%CI)	P[b]
Age at last full term pregnancy (in years)					
≤24	145 | 498	**Reference**		**Reference**	
25-29	262 | 645	1.26 (0.98-1.61)	0.061	1.04 (0.77-1.41)	0.751
30-34	231 | 353	1.84 (1.41-2.41)	≤0.001	1.31 (0.94-1.41)	0.104
≥35	130 | 116	2.45 (1.74-3.43)	≤0.001	1.49 (0.97-2.30)	0.068
Missing	22 | 114				
P trend			≤0.001		0.028
Oral contraceptive use					
Never	732 | 1,560	**Reference**		**Reference**	
Ever	47 | 140	1.03 (0.72-1.48)	0.851	1.01 (0.64-1.58)	0.951
Missing	11 | 26				
Age at oral contraceptive use started(in years)					
Never	732|1,560	**Reference**		**Reference**	
≤24	19|64	1.03 (0.60-1.76)	0.912	0.98 (0.51-1.90)	0.973
25-29	11|48	0.61 (0.31-1.22)	0.167	0.80 (0.36-1.75)	0.58
≥30	14|26	1.59 (0.79-3.17)	0.187	1.04 (0.39-2.75)	0.934
Missing	14|28				
P trend			0.85		0.773
Total duration of oral contraceptive use (in months)					
Never	732|1,560	**Reference**		**Reference**	
<13	21|70	0.95 (0.56-1.59)	0.846	0.80 (0.42-1.52)	0.514
13-49	18|41	1.37 (0.76-2.47)	0.281	1.72 (0.85-3.46)	0.127
>49	5|28	0.47 (0.17-1.28)	0.142	0.29 (0.07-1.14)	0.077
Missing	14|27				
P trend			0.663		0.598

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), education(less than 5 years schooling , ≥5 year of education),current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), education(less than 5 years schooling , ≥5 year of education), current residential region(north, north-east, central, west, south), gallstone(present/ not present), waist to hip ratio (continuous), tobacco chewing and tobacco smoking (yes/no), number of full term pregnancies

[c]same as b. However, adjusted for total number of pregnancies(continuous) instead of number of full term pregnancies

Missing values were excluded from analysis

Table 2.25 Odds ratios and 95% confidence intervals for gallbladder cancer in relation to joint effect of number of full term pregnancy, age at full term pregnancy and age at menarche

Parameters	Categories	Case/Control	OR[a] (95% CI)	p-value	OR[b] (95%CI)	p-value
Number of full term pregnancy and age at first full term pregnancy(years)	0 to 1 & ≥24	28 | 139	Reference		Reference	
	2 to 3 & ≥24	85 | 282	1.21 (0.73-2.00)	0.442	1.10 (0.58-2.09)	0.745
	≥4 & ≥24	53 | 43	2.43 (1.23-4.80)	0.011	2.57 (1.11-5.95)	0.027
	0 to 1 & ≤23	17 | 60	Reference		Reference	
	2 to 3 & ≤23	212 | 716	0.82 (0.46-1.45)	0.504	0.99 (0.47-2.06)	0.984
	≥4 & ≤23	375 | 381	1.78 (0.99-3.21)	0.052	2.02 (0.95-4.28)	0.064
Number of full term pregnancy and age at menarche (years)	0 to 1 & ≥16	3 | 31	Reference		Reference	
	2 to 3 & ≥16	51| 146	2.85 (0.82-9.86)	0.098	1.12 (0.30-4.11)	0.862
	≥4 & ≥16	74 | 72	5.50 (1.53-19.74)	0.009	2.17 (0.56-8.36)	0.257
	0 to 1 & ≤15	5 | 55	Reference		Reference	
	2 to 3 & ≤15	101| 278	1.06 (0.72-1.56)	0.745	1.25 (0.77-2.02)	0.354
	≥4 & ≤15	161 | 150	2.34 (1.56-3.52)	≤0.001	2.68 (1.61-4.45)	≤0.001

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratios

[a]Adjusted for age(continuous), education(less than 5 years schooling , ≥5 year of education),current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), education(less than 5 years schooling , ≥5 year of education), current residential region(north, north-east, central, west, south), gallstone(present/ not present), waist to hip ratio (continuous), tobacco chewing and tobacco smoking (yes/no), number of full term pregnancies

Missing values were excluded from analysis

Table 2.26 Summary of findings

Variables	Categories	Odds ratio(95%CI)	P value
Birth place	Birth place in high risk region vs birth place in low risk region	4.82(3.87-5.99)	≤0.001
Gallstone history (self-reports)	Gallstone present vs Gallstone not present	28.94(21.55-38.96)	≤0.001
Gallstone history (stringent definition)	Gallstone present	6.10(4.34-8.56)	≤0.001
Tobacco smoking	Bidi smoker vs non-smoker	1.87(1.28-2.73)	0.001
	Cumulative years of bidi smoking (>400) vs non-smoker	2.82(1.49-5.33)	0.001
Smokeless tobacco	Tobacco quid chewing/day (>5 times vs non-chewer)	1.60(1.17-2.20)	0.003
	Tobacco quid chewing duration (>19 years vs non-chewer)	1.43(1.05-1.94)	0.02
	Betel leaf with Tobacco quid chewer vs non-chewer	1.55(1.12-2.13)	0.007
	Betel leaf with Tobacco quid chewing/day (>5 times vs non-chewer)	2.61(1.40-4.88)	0.003
	Betel leaf with Tobacco quid chewing duration (>19 years vs non-chewer)	1.60(1.08-2.37)	0.019
Obesity (Men)	Waist to hip ration(≥0.97 vs ≤0.90)	1.93(1.30-2.86)	0.001
	Body size at age 10(≥5 vs <3)	2.00(1.35-2.96)	≤0.001
	Body size at age 20(≥5 vs <3)	2.3(1.45-3.69)	≤0.001
Obesity (Women)	Waist to hip ration(≥0.95 vs ≤0.84)	4.68(3.20-6.86)	≤0.001
	Body size at age 10(≥5 vs <3)	2.52(1.90-3.34)	≤0.001
	Body size at age 20(≥5 vs <3)	2.98(2.10-4.23)	≤0.001

continue…

Variables	Categories	Odds ratio(95%CI)	P value
Dietary factors	All fruits (Highest quartile vs lowest quartile)	0.53(0.41-0.69)	≤0.001
	Citrus fruits (Highest quartile vs lowest quartile)	0.63(0.47-0.83)	0.001
	Non-Citrus Fruits (Highest quartile vs lowest quartile)	0.55(0.42-0.70)	≤0.001
	Any vegetable (Highest quartile vs lowest quartile)	0.63(0.49-0.80)	≤0.001
	Leafy and green vegetables (Highest quartile vs lowest quartile)	0.66(0.52-0.85)	0.001
	Root vegetables (Highest quartile vs lowest quartile)	0.75(0.59-0.96)	0.022
	Fruiting vegetables (Highest quartile vs lowest quartile)	0.60(0.47-0.76)	≤0.001
	Cruciferous vegetables (Highest quartile vs lowest quartile)	0.68(0.53-0.87)	0.003
	Tomatoes (Highest quartile vs lowest quartile)	0.74(0.57-0.95)	0.021
	Onion and Garlic (Highest quartile vs lowest quartile)	0.67(0.52-0.85)	0.001
	All Cereals (Highest quartile vs lowest quartile)	0.69(0.54-0.89)	0.005
	Fenugreek (Highest quartile vs lowest quartile)	1.91(1.5-2.43)	≤0.001
	Mustard Oil (Highest quartile vs lowest quartile)	3.59(2.96-4.37)	≤0.001
Reproductive factors	Number of full term pregnancies (≥4 vs 1)	2.34(1.46-3.74)	≤0.001
	Age at menarche (15-20 years vs ≤12)	1.61(1.09-2.36)	0.015

2.8 Discussion

The present study is the one of the largest case-control studies conducted globally to understand role of lifestyle factors and infection with *H. Pylori* in aetiology of GBC. The study was conducted in India, which has one of the highest incidence rates of GBC and in TMH, Mumbai with inflow of cases from every regions of India for diagnosis and treatment. Detailed questionnaire-based data were collected on various lifestyle risk factors such as birth place, past medical history such as gallstone, tobacco habits, dietary intakes, and reproductive histories. Detailed anthropometric measurements were also recorded for all study participants. The main goal of the analysis was to investigate risk factors for GBC in India.

The study was unique in the following ways:

1. One of the largest case-control studies of GBC with enrolment of 1,170 cases and 2,525 controls.
2. The data were collected by trained staff with stringent quality control checks at three levels viz: immediately after data collection, at the time of data entry in electronic database and logical checks at the time of analysis.
3. The interviews were conducted in a closed room with 10% of individuals re-interviewed on selected variables to assess the reproducibility of response. Good correlation (in the range of 80 to 100%) was obtained in responses.
4. The selection of cases was done using stringent criteria with all cases microscopically confirmed (either by histology of primary or with cytology or histology of secondary site with radio imaging data supportive of primary GBC).
5. The controls were enrolled carefully so as to sample them from same study base. Thus controls were enrolled from all the DMGs of TMH, Mumbai. Not more than 20% controls were selected from a single DMG. Further, the enrolled visitor controls were

friends, neighbours, spouse and relatives. The controls from gastrointestinal DMG were not included in the study. Controls were enrolled concurrently to the cases.

2.8.1 Place of birth

The geographical differences in rates of GBC globally and within India are well known. However there have been no analytical studies to date evaluating the effect of place of birth and effect of migration from high to low risk region on risk of GBC. Using geographical differences in rates of GBC within India, we divided the participants into high risk (with high incidence rates of GBC) and low risk (with low incidence rates of GBC) regions and demonstrated using a case-control study approach that place of birth in high risk region is associated with increased risk of GBC even after adjustment for potential confounders and that the risk is retained even after migration from high risk to low risk region.

This data suggest that GBC has both environmental and genetic etiology, and that differences in rates of GBC may be explained by studying these lifestyle related factors. Further, a large proportion of GBC cases residing in low risk areas were born in a high risk region (44.81%), suggesting that moderately higher rates in some of low risk areas (such as Mumbai) might be at least partially due to migration of population from high risk region (particularly from Bihar and North India).

2.8.2 Gallstone history

Gallstone is an important risk factor for the development of GBC; however, not all individuals with gallstone develop GBC. It has been observed that countries with higher prevalence of gallstone disease tend to have higher incidence of GBC . In the current study to minimize the effect of misclassification of gallstone, gallstone history was ascertained using two different definitions based on duration of gallstone diagnosis. We observed that prevalence was higher among study participants residing in north, north-eastern regions using

both definitions of gallstone history. We observed very high odds ratio (around thirty fold) for developing GBC in individuals with history of gallstone. However, when we estimated odds ratio using a stringent definition for history of gallstone, the odds ratio for developing GBC was reduced to seven fold. Significant reduction in risk was observed with increase in duration of gallstone history using both definitions. Although the statistically significant association observed in our study is biologically plausible; as chronic inflammation due to gallstone particularly of larger size may cause metaplastic and dysplastic changes; there are difficulties in declaring the association with gallstone as causal. It is possible that gallstones are detected more in cases compared to controls because of investigative procedures (computed tomography (CT) scan, Ultrasound scan (USG) which are frequently carried out for cases. Self-reported ascertainment of gallstone thus may have resulted into differential misclassification of study participants. The differential misclassification may have also been resulted because of cases recalling history of gallstone disease more often than controls. Studies conducted in other parts of world and smaller studies from India (104–107)also similarly observed increased risk of GBC with history of gallstone, however, all these studies encountered similar problems of differential misclassification. Studies in future should plan to conduct some imaging procedures among controls so that diagnosis of gallstone is valid and also comparable among cases and controls. Future studies should also classify gallstone by size and types. The association observed in current study and many other studies in literature suggest that gallstone might be a risk factor for GBC, and there is a need to confirm this association using detailed studies with imaging done on controls and details of type of the stone.

2.8.3 Tobacco habits (tobacco smoking and smokeless tobacco)

Smoking tobacco: From the available surveys at national level, bidi smoking appears to be predominant form of smoking in most parts of India (108). The current study also indicated that bidi smoking is also most predominant form in study population and is particularly prevalent among men **[Table: 2.9].** We therefore conducted detailed analysis on smoking for all study participants and no gender specific analysis was performed. Results from the current study provide good evidence that bidi smoking is a risk factor for GBC, as the elevated risk was observed in a dose-response manner with increase in number of bidi smoked, duration of bidi smoking and cumulative years of bidi smoking (by multiplying number with duration). However, study observed weaker association for ever smoker. Ever smoker is comprised of different forms of tobacco smoking including cigar, cigarettes, pipes, dhumati etc), which was used typically for small duration and less number smoked per day. This could have diluted the risk between ever smoker and GBC.

This is the first study to identify the role of bidi smoking in GBC. The results are biologically plausible given the role of bidi smoking in various other cancer sites (lung, esophagus, larynx, mouth, throat, kidney, bladder, liver, pancreas, stomach, cervix, colon, and rectum). Bidis are hand-rolled; their tobacco content varies considerably in individual sticks. Typically, an individual bidi may contain roughly 0.15 to 0.5 gram of pulverized sun-cured locally grown tobacco in a tendu or temburi leaf obtained from native plants, *Diospyros melanoxylon or Diospyrus ebemum*, respectively. Many chemicals in tobacco smoke are known to have toxic properties, as discussed below, and some (E.g. nicotine) may result in addiction. Mainstream smoke from bidis contains many potentially harmful chemical constituents, including carcinogenic chemicals such as the tobacco-specific nitrosamines (TSNAs), polyaromatic hydrocarbons (PAHs), aromatic amines, phenols, and metals. These

metals may get stored in gallbladder, causing constant irritation to gallbladder mucosa and initiating carcinogenesis. The plant species used for leaf wrapper (*Diospyros malanoxylon, Diospyros ebenaster, Diospyros ebenum, and Diospyros isamlii)* contains naphthoquinones and coumarins, but not nicotine. In addition to contributing heavily to the overall tar delivery, the leaf wrapper may generate harmful compounds. The TSNAs, N-Nitrosonornicotine (NNN) and Nicotine-derived nitrosamine ketone (NNK), present in bidi tobacco and bidi smoke are categorized as reasonably anticipated to be human carcinogens by the National Toxicology Program(109). It has been shown that(110)tobacco smoking causes non-significant prolongation of gallbladder emptying time in smokers, delays gallbladder contraction and decreased gallbladder emptying volume. Possible mechanisms of action include disruption of gallbladder smooth muscles contraction or, decreasing cholecystokinin release via inhibition of intestinal motility. In humans, this chronic process is associated with gallstone formation which is a strong risk factor for GBC.

Smokeless tobacco: This is the first study to demonstrate the effect of chewing tobacco (form of smokeless tobacco) as well as smokeless tobacco on GBC risk. Chewing tobacco in the form of tobacco *quid* alone or with betel leaf was observed to increase the risk of GBC. Chewing tobacco *quid* with or without betel leaf showed an increase in risk with number of *quid* chewed per day as well as with duration of chewing. The data also suggest that less consumption of smokeless tobacco may not be associated with GBC or has a small effect which could not be detected because of less power**[Table 2.12, Table 2.13].**. It is possible that consumption behaviour of smokeless tobacco for those chewing less tobacco (i.e. ≤5 times/day) is different from those consuming more tobacco(i.e. >5) particularly in regard to duration of placement of tobacco *quid* and spitting affecting the risk for development of GBC. However, the study observed strong risk of developing GBC with high consumption of

any type of smokeless tobacco, also with long duration of use suggesting possible association between smokeless tobacco and GBC. These observations are biologically plausible as chewing tobacco is associated with many other cancer types such as oral cavity and oropharyngeal cancer. Further, chewing tobacco results in exposure to tobacco-specific nitrosamines, N-nitrosamino acids, ,volatiles aldehydes, such as formaldehyde and acetaldehyde, and exposures to metals including cadmium, lead, arsenic, nickel, and chromium, and radioactive elements(111). It has been hypothesized that heavy metals get stored in gallbladder, dissolved in the bile, and caused irritation to gallbladder mucosa and may initiate carcinogenesis as hypothesized.

This finding of tobacco consumption and increased risk of GBC has major public health significance as smokeless tobacco use is a major form of tobacco use in India.

2.8.4 Anthropometric measurements

We observed positive associations between GBC and measures of central obesity viz : WHR, and waist circumference. For WHR, the association clearly persisted even after adjustment for BMI for both men and women. The measurements were done twice and by trained investigators. The measurement of waist circumference in cases was however bit difficult because of ascities, and we could not accurately measure the waist circumference for 186 cases (15.86%). All these 186 cases were excluded from analysis of waist to hip ratio.

Being overweight or obese during childhood and early adulthood as determined by pictorial images of body sizes (at childhood and adulthood) was significantly associated with elevated risk of GBC. Although recalling the body size during different periods of life might have its limitations , the method has been successfully used in many prospective and case-control studies(78,112,113). In addition to affecting lipid metabolism , obesity can affect the risk of gallstones and GBC through the adverse changes in the hormone-binding globulin, insulin

growth factor –I , and inflammatory mediators , such as insulin and cytokines; all of these stimulate proliferation and inhibit apoptosis , thereby enhancing the potential for tumour growth(78) .

Study observed protective association between height and gallbladder cancer risk, contrary to that Norwegian cohort study (could not observe any association between GBC and height). Given, the association of height with several other cancer types (breast, colon) this finding could be of interest. As GBC is associated with low-socio economic status, the observed association could well be due to residual confounding, however the possibility of genetic factors or some exposure at childhood in explaining this association cannot be ruled out.

We observed an inverse association between BMI and GBC. The inverse association from BMI, however, may be artefact, as all GBC patients lose weight because of the disease. Thus observed inverse effect of BMI on GBC most likely attributed to reverse causality because of preclinical effects of cancer on body weight such as cachexia or body wasting(114). It is important however, to note that although there was reduction in overall body weight because of GBC, reduction in abdominal fat affects to have been less and we could observe a strong risk for central obesity.

Study observed strong association between central obesity and GBC. Given the strong biological plausibility, and similar results from previously conducted large scale studies(98),central obesity has strong potential for public health intervention to reduce burden of GBC.

2.8.5 Dietary intake

The current study identified several protective food groups (vegetables, fruits) as well as foods associated (fenugreek, fresh fish, and mustard oil) with increased GBC risk. These findings are consistent with previously reported studies on GBC and diet (38). The protective

association of fruits and vegetables has been observed for many other cancer sites (115). Given the biological plausibility and similar protection observed from experimental studies, observed protective association for fruits, and vegetables might be real. Fruits, vegetables, and whole grains contain a wide verity of antioxidants compounds (photochemicals), such as flavanoids, and caretenoids(116). These nutrients and others in diet have potential to prevent cancer, by either protecting against oxidative stress and/or DNA damage, or inhibition of cell proliferation and oncogene expression(117). The protective association for allium and garlic observed in present study is plausible given the beneficial nature of organosulfer compounds present in allium and garlic. Protective role of allium and garlic has been observed for many other cancer sites including stomach, colon and oesophagus (118).

High consumption of tomato was observed to be protective in the current study. The protective effect of tomato intake could be due to the presence of the fat-soluble pigments: lycopene. Population studies have shown that high intake of lycopene is inversely associated with the incidence of certain types of cancers, including those of the digestive tract, prostate, and cervix(119).

Finding showing increase in risk of GBC with highest consumption of fenugreek were unexpected given the use of fenugreek as a local medicine for treatment of reducing blood sugar and cholesterol levels (120). Animal models have demonstrated gallstone prevention by using extracts of fenugreek (121,122). However, we are not sure whether increased in risk because of some unmeasured confounding or confounding association between mustard oil and GBC, due to intake of adulterated fenugreek in high risk region. The adulteration of fenugreek may be likely responsible for increase in the risk of GBC, in the same way use of analogy to the *Aristolochia* (a herbal medicine used in china) to be associated with uroepithelial malignancies(123).

One of the most important findings of current study, which could have public health potential if replicated, is the observed association of high intakes of mustard oil with GBC. The risk was extremely strong with highest consumption, showing a four-fold increase in a dose response manner. As use of mustard oil is more common in North-India(124), a region with high incidence of GBC, we did stratified analysis to observe effect of mustard oil use in high and low risk incidence region. The statistically significant increase risk persisted in both high and low risk regions. This possibly indicates that the results are less likely due to selection bias. A strong fourfold increase for mustard oil use, encounters any argument for association to be confounded by other variables. It has been observed that mustard oil has an inflammatory response(125). This commodity has also attracted maximum scope for adulteration with argemone oil and butter yellow dye. Sanguinarine and diethynitrosamine are found to be in adulated in fried mustard oil. These components interact with macromolecules like DNA and proteins to initiate genotoxic and mutagenic response. Further, these compounds have been found to produce hepatic and skin tumors as well as cancer in respiratory tract. Study investigating the role of mustard oil on development of GBC found a high concentration of sanguinarine and diethynitrosamine in gallbladder tissue of GBC patients compared to cholelithiasis patients suggesting an association with GBC. However, further studies are needed to understand the association between mustard oil and GBC (126).

The current study similarly observed the association of GBC with high consumption of fresh fish but not with dry fish. Again, fresh fish consumption is more common in North and North-East India. However, sensitivity analysis revealed the statistically not significant increased association in both high and low risk regions. It is possible that the association with fresh fish could be due to some infectious agent which might get into human by fresh fish

intake. The most common suspected infectious agents to be investigated are *Opisthorchis viverrini*, *Opisthorchis, felineus*, and *Clonorchis sinensis*(127).

Study did not observe any association with intake of meat, chicken, all spices combined and risk of GBC.

Even though the results observed for dietary variables in current study are quite strong, interpretations of dietary results have inherent problems in case-control study designs. Timing of exposure capture is probably the most important reason for using a cohort versus a case-control study design for examining dietary exposures. Cancer is a multiphase and multifactorial disease that essentially occurs late in life but that might be affected by different (early) exposure window, which is difficult to evaluate using case-control study design. Furthermore; it is difficult to quantify the dietary intake and the design of food frequency questionnaire (FFQ) used in current study may not be adequate for participants coming from different parts of India. An attempt was made to include most common food items in the questionnaire. Present study FFQ contained about 77 dietary items including fruits, vegetables, spices, pickles and cooking oil. We believe that even if there is some misclassification it is non-differential and will thus pull the effects towards null. Future studies should include detailed and more precise information particularly on use of mustard oil, consumption of fresh fish and consumption of fruits and vegetables.

2.8.6 Reproductive and hormonal factors:

In agreement with other studies (41, 63, 65, 67, 68, 68), the current study showed positive association of GBC with number of full term pregnancies. The joint effect evident for parity and early age at first full term pregnancy was similar to that observed in a study from China(63). Similarly, findings of the current study in terms of a significant positive association with late age at menarche is consistent (41,63).These associations for increased

risk of GBC in relation to reproductive factors indicate a possible role of endogenous hormones in disease aetiology. Exogenous hormones, mainly in the form of use of oral contraceptive however showed no association with GBC risk in current study.

2.9 Summary:

The current study is the first large scale study to report the role of several lifestyle related variables such as tobacco habits, female reproductive factors, dietary intake with respect to the risk of GBC. Past medical history such as gallstone history has been studied for its association with risk of GBC. These findings are summarized below:

1. Birth place in a GBC high risk region is a major risk factor for development of GBC. Risk persisted in individual, who migrated from high to low risk regions. The majority of GBC cases were born in high incident region which is comprised of Bihar, Delhi, Himachal Pradesh, Punjab, Chandigarh, Rajasthan, Uttarakhand, Uttar Pradesh, Assam, Tripura, Sikkim, Jharkhand, and West Bengal.
2. History of gallstone is a risk factor for GBC. Future studies should be designed to answer the question of temporality of association as well as to understand the type and size of gallstone in increasing the risk of GBC. History of gallstone among controls needs to be ascertained by imaging diagnostic modality to validate self reported history, so that exposure information is comparable among cases and controls.
3. Tobacco consumption in the form of bidi smoking or tobacco *quid* chewing with or without betel leaf increases risk of GBC.
4. We identified that central obesity is an important factor associated with increased risk of GBC.

5. A diet rich in fruits and vegetables particularly allium vegetables was observed to be strongly protective against GBC. High intake of fenugreek and fresh fish consumption was found to be associated with increased risk of GBC. However, further studies are required to confirm the findings.
6. Risk of GBC increases with number of full term pregnancies. Women who have given birth to 4 or more children have a 2.34 fold risks of GBC compared to women who have not given single birth. Women who attain their age at menarche after 14 are 1.34 fold risk of developing GBC.

Chapter 3 : *Helicobacter pylori* and gallbladder cancer

3.1 Introduction

Helicobacter pylori (H. pylori) is a gram negative, microaerophilic bacterium which can colonize the stomach and cause peptic ulcer disease and gastric cancer(48), making it the fifth most common malignancy in the world(33).

More recently, a growing body of literature supports the possible role for *H. pylori,* and possibly other *Helicobacter* specie, in extragastric cancers. Molecular studies using polymerase chain reaction assay (PCR) have detected bacterial DNA of *helicobacter* species, in bile fluid and tissue from patients with gallstone and GBC(38). The relative risk (RR) produced are consistent and range from 5.9 to 9.9 among studies reported in literature. These findings are supported by studies conducted using animal model and could be biologically plausible. Mice studies have reported that *Helicobacter* species facilitate gallstone formation and biliary tract tumours (128).*In vitro*, the urease produced by some *Helicobacter* species, including *H. pylori,* can precipitate calcium salts(129), potentially contributing to gallstone formation and biliary tract cancer. Also in mice, *Helicobacter hepaticus* can colonize other organs of the gastrointestinal tract and cause liver tumours (130–132). Despite evidence that *helicobacter* species promote gallstone formation and hepatobiliary tumours in laboratory studies, it remains unclear if *helicobacter* species contribute to GBC. Further research is required on *Helicobacter* species, as this could be a means of prevention of GBC.

Several techniques, both invasive and non-invasive, are available for diagnosing *H. pylori* infection. Invasive methods require the collection of pinch biopsy samples taken during diagnostic procedures such as upper gastrointestinal endoscopy. Using these biopsies, *H. pylori* is identified by either culture or histological examination or urease testing. Limitations with these invasive techniques are that they are tedious to perform and time consuming. They also require sampling procedures that may cause patient discomfort.

Hence in contrast to cross-sectional tissue-based approaches, study used *H. pylori* seropositivity to determine infection of *H. pylori* and its association with GBC. This chapter focuses on serological analysis of *Helicobacter* species particularly *H. pylori,* and associated risk of GBC in a large scale epidemiological study design.

3.2 Study population and specimen procurement

GBC cases and visitor controls enrolled in the study were frequency matched on age (± 10 years), gender, and region of current residence at the time of enrolment (North, North-east, south, central, south). Other methodological details are described in Chapter 2. The serological study included 1,764 study participants comprising of 859 GBC cases and 905 controls. Final analysis was performed on 833 GBC cases and 818 after quality control assessment.

A blood sample was collected by venepuncture using a serum separator tube/non-gel serum tube. Serum was fractionated by centrifuging blood at 1,500 r.p.m. for 10 minutes, immediately after clotting of blood sample for 30 minutes prior to centrifugation and fractionation. Two serum aliquots (each of 2000ul) were stored at -80°C in sterile, pre-labelled cryo-vials for long term storage purpose. Repeated freeze-thaw cycles and delay in centrifugation step was avoided to ensure quality of serum sample. We did not perform the assay on highly lipemic serum samples.

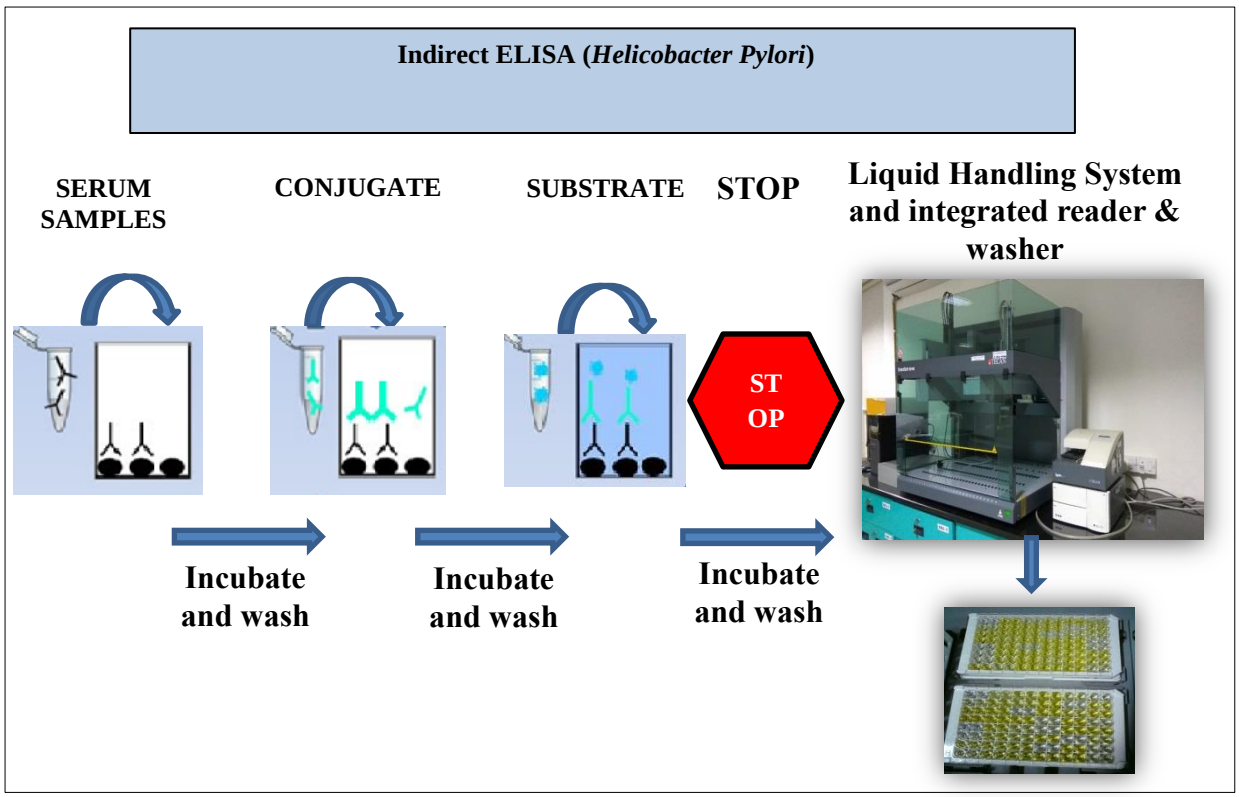
3.2.1 Strategies for investigation of *Helicobacter pylori*

Commercially available Pyloriset EIA-G III (Orion Corporation, Orion Diagnostica, Espoo, Finland) was used to determine *H. pylori* IgG antibodies in serum of study participants. The Pyloriset EIA-G assay determines *H. pylori* IgG antibodies titre in serum by means of indirect enzyme linked immunosorbent assay (Indirect ELISA). The assay was conducted by coating mico-titre wells with *H. pylori* specific antigens followed by addition of dilutions of

serum samples and calibrator serum /reference standards. The *H. pylori* specific IgG antibody bound to the *H.Pylori* specific antigen coated on mico-titre well plate was detected with horseradish peroxides conjugated anti-human IgG, followed by the addition of the substrate and development of characteristic chromophore. The absorbance of each well was measured at 420 nm using ELISA plate reader.

The reported sensitivity and specificity of the assay are 92% and 84% respectively (133). ELISA was performed using automated liquid handling system integrated with micro-titer plate washer and reader.

Figure 3.1: Illustration of Indirect ELISA



3.2.2 Quality Control measures

An appropriate workflow plan was established for implementation of the ELISA protocol to ensure optimal results. As a part of this workflow, stringent quality control measures were followed at various stages of assays, which included laboratory operations, and inclusion of quality control serum samples in every assay and assay validity criterion.

3.2.2 A. Laboratory operations:

All the assays were performed using Good Laboratory Practices (GLP). Laboratory operations involved, buffers preparations/handlings, equipment calibration and monitoring/ maintenance of laboratory temperature/humidity. Sterile filters, containers and sterile, depyrogenated reagent grade water were used for reagents and buffers preparation. All glassware used for preparing buffers/solutions were washed and rinsed with pyrogen free water, depyrogenated by heating in an oven at 180°C for minimum 2 hours to remove endotoxin, or endotoxin free plastic was used. All containers for solutions were labelled with the reagent name, date prepared, name of preparer, and expiration date. All reagents/buffers and plastic/glassware were stored aseptically at required temperature. All reagents and antigen-coated plates were equilibrated at room temperature prior to use to reduce variability in daily performance. Before use, buffers/solutions were checked for signs of any contamination, which may include flocculence, unusual colour or cloudiness. The lot number of ELISA kit was recorded to access the batch effect. Cleaning, sterilization and depyrogenation of supplies were documented for investigations of atypical results or unacceptable rate of invalid assays.

All equipment, particularly automated liquid handling platform was periodically monitored for maintenance and calibration purpose for expected performance. Routine maintenance of

microtiter 12 well washing device was carried out using of 0.5 M NaOH, followed by sufficient rinse (5 times) with deionized water as per manufacturer's recommendation.

All assays were performed at controlled conditions to avoid variation in day-to-day performance of ELISA methods. Control conditions involved maintenance of laboratory temperature at 25°C with relative humidity of 70-75%.

3.2.2 B. Quality Control (QC) serum samples

Quality control serum samples were in-house serum samples used to monitor inter-assay variation as well as intra-assay performance. All QC samples had known ranges of acceptable results, which were obtained by 10 assays on each serum sample. The range represents mean ± 2 standard deviation (SD) of antibody levels determined in 10 assays. Two QC samples (one positive & one negative) were included in each plate to assure accuracy of results.

3.2.2 C. Assay validity criterion

Serum samples were tested in duplicate on micro-titer well plate. If the absorbance values of replicates/duplicates had a coefficient of variation (CV) > 7 %, the concentrations obtained with the absorbance data for that dilution was excluded from the calculation of the antibody concentration of the sample. Quality control sample values were checked for established range (which is ± 2 SD of mean), or the plate was rejected and samples re-analyzed. The absorbance reading of the calibrator serum /reference standard 1 and 4 were checked for their absorbance, which should be below 0.200 and at least 0.800 respectively, or else assay plate was reanalyzed. Higher dilution (greater than 1:200) was used if a sample has absorbance readings greater than that of 2.0(predefined limit of detection, as per manufactures instructions)

3.3 Procedure

3.3.1 Preparation of reagents

Washing buffer was diluted 10 times before use with distilled or deionized depyrogenated water. Serum dilution buffers, enzyme conjugate, calibrator sera/reference standard, TMB-substrate, stopping solution were ready to use.

3.3.2 Serum sample dilution

Serum samples were diluted 1: 200 times in serial dilution with serum diluting buffer. Serum samples were first diluted to 10 times, followed by additional 20 times, making the final dilution 1 is to 200 times.

3.3.3 Plan the assay and sample layout

The assay was planned for number of plates to be used depending upon the number of unknown serum samples. Samples were tested in duplicates on a micro-titer well plate. GBC cases and control samples were planned in alternate manner. Plate layout was planned as per described in plate layout Figure 3.2.

Figure 3.2Typical microtiter plate layout for H.pylori elisa

	Standard/QC samples		Unknown Case		Unknown Control		Unknown Case		Unknown Control		Unknown Case & Control	
A	Cal1*	Cal1	3	4	5	6	7	8	9	10	11	12
B	Cal2	Cal2										
C	Cal3	Cal3										
D	Cal4	Cal4										
E	QC sample1δ	QC sample1										
F	QC sample1	QC sample1										
G	Unknownμ	Unknown										
H	Unknown	Unknown										

*: Calibrator/reference standard, δ: Quality control samples, μ: Unknown; test serum sample

3.3.4 Assay methodology

The assay was performed using automated liquid handling platform with robotic arm(TECAN Freedom EVO100), integrated with automated ELISA microtiter plate reader (TECAN Sunrise reader) with 405 nm filter and microtiter 8 well washing device(TECAN HydroFlex washer). A protocol program was written and standardized for conducting ELISA assay using appropriate software (Freedom EVOware software).

Sample incubation

- 100ul of calibrator Serum/reference standard (1-4) were added to designated wells of first column, as per plate layout
- 100ul of diluted test serum samples were added to designated wells as per plate layout
- Plates were incubated on plate shaker at 18-25°C for 30 minutes at 700 rpm
- Each well was aspirated and washed 3 times with washing buffer using automated micro-titer 8 well plate washing device, which described in washing procedure

Conjugate incubation

- 100ul of Enzyme Conjugate was added into each plate
- Plate was incubated on plate shaker 18-25°C for 30 minutes at 700 rpm
- Each well was aspirated and washed 3 times with washing buffer using automated micro-titer 8 well plate washing device, which described in washing procedure

Substrate incubation:

- 100ul of substrate was added into each plate
- Plate was incubated on plate shaker 18-25°C for 10 minutes at 700 rpm
- 100ul of stopping solution was added into each well, to stop the enzyme reaction

Absorbance measurement

- Micro-titer plate reader was (photometer) was blanked without plate (air blank).

- Absorbance of each well was read at 405nm, within 10 minutes after stopping the reaction.

Washing Procedures:

An automated micro-titer plate washing device integrated liquid handling platform was used. Wells were completely filled with washing buffer, 300 ul of washing buffer for washing purpose.

3.4 Analysis

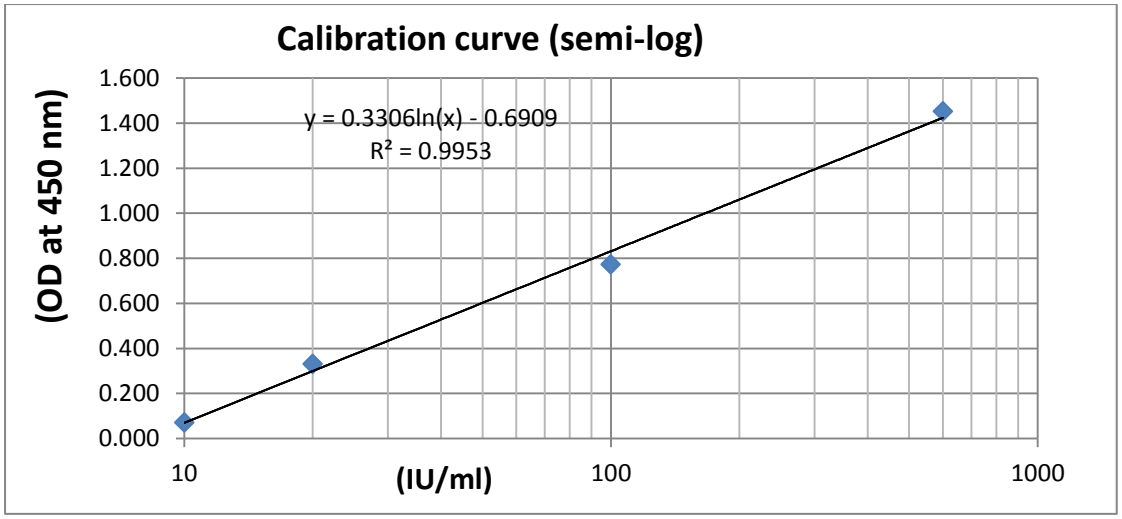
An automated reader, with appropriate software program (Magellan^{TM}) was used for calculation of results. Absorbance data were converted to antibody concentration using the software program Magellan^{TM} for interpretation. The *H. pylori* IgG 4 antibody concentration for each sample was calculated based on a standard curve generated from the reference/calibrator standards on the plate. Reference/calibrator standards were run every time on micro-titer well plate. A point to point calibration line on a semi-logarithmic scale using the absorbance of the reference/calibrator standards: the units of the calibrators on the x-axis (logarithmic) and the respective absorbance on the y-axis **[Figure 3.3]** was generated using program software. Best fitted calibration line was obtained using linear regression analysis:

- The slope of best fit line was in the range of 1 ± 0.2
- The intercept was always <0.1
- The correlation of determination "r^2" was always >0.95.

The absorbance readings are proportional to the logarithm of the antibody concentration (IU/ml). Mean absorbance reading of the calibrators/reference standard and patient serum samples were calculated. Unit value of patient serum was read using a best fit calibration line. If the absorbance of the sample was higher than that of the highest Calibrator Serum, i.e. the

units could not be read on the calibration lines, the sample was tested once again; using a higher dilution, and the unit of value achieved with this dilution was multiplied by appropriate dilution factor.

Figure 3.3 Mean absorbance reading of the calibrators/reference standard vs concentration (IU/ml)



3.5 Interpretation of ELISA assay results

If the IU/ml or absorbance of the serum sample was equal to or higher than that of the Calibrator serum/reference standard 2, the result was considered to be positive for *H.Pylori* IgG antibodies. If the IU/ml or absorbance of the serum sample was lower than that of the Calibrator serum/reference standard 2, the result was considered to be negative for *H.pylori* IgG antibodies.

Table 3.1 Interpretation of *H.pylori* serological assay

Pyloriset EIA-G III(Result , IU/ml)	**Interpretation**
≥ 20	Positive for *H.pylori* IgG antibodies
< 20	Negative for *H.pylori* IgG antibodies

3.6 Statistical analysis:

Gender specific ORs and their 95% Confidence interval for GBC, based on results of the *H.Pylori* IgG antibody assay, were derived using the unconditional logistic regression method. Unconditional logistic regression models were adjusted for the following potential confounders : age (continuous variable), region of current residence (North, South, North-East, West and Central India), education (Less than 5 years of schooling, ≥5 year of education), WHR (continuous variable), gallstone history(present, not present), tobacco chewing(yes/no), and tobacco smoking (yes/no).In order to avoid, arbitrariness in selection of cut-off points , study participants were classified into four groups of increasing antibody titters ,using quartiles of the controls distribution as cut points. Test for linear trend for ordered variables were performed by assigning the score j to the j^{th} exposure level of a categorical variable (where j = 1, 2 . . .) and treating it as a continuous predictor in unconditional logistic regression. Analysis was performed on non –missing values and study participants with missing value for one or more of the variables in statistical model were eliminated from analysis..

3.7 Result

A total of 859 GBC cases and 905 controls were tested for *H.pylori* IgG antibody. Demographic characteristics of the study participants are presented in Table 2.4.

3.7.1 Quality Assessment

All serum samples were tested in pair-wise duplicate. Absorbance values obtained from 113 samples were excluded based on cut off value of 7% CV. Final analysis was conducted on 833 GBC cases and 818 controls after quality control assessments.

3.7.2 Prevalence of IgG antibodies to *H.Pylori*

Overall, the prevalence of *H.pylori* IgG seropositivity was 81.01% in men and 80.52% in women GBC cases and 74.81% and 78.93% among matched men and women controls respectively. *H.pylori* IgG seroprevalence tended to be higher in GBC cases and controls with slightly lower seroprevalence in the age-group of 60+ compared to 50-59 age group**[Table 3.3]**.The prevalence of *H.pylori* IgG was approximately 80% in all five geographical regions of India**[Table 3.4].**

3.7.3 *H.pylori* IgG antibody and GBC risk

Overall, in an unconditional multivariate model taking into account age (continuous variable), region of current residence (North, South, North-East, West and Central India), education (Less than 5 years of schooling, ≥5 year of education), WHR (continuous variable), Gallstone history(present, not present), tobacco chewing(yes/no), and tobacco smoking (yes/no), *H. pylori* seropositivity was not associated with risk of GBC ($OR_{\text{All study participants}}$=1.07, 95% CI: 0.81-1.43)**[Table 3.5].** Moderate but non-significant risk was observed for men in the gender specific analysis (OR_{Men}=1.81, 95% CI: 0.96-3.42)

[Table 3.5].

Table **3.5** shows the quartiles of the control distribution using the index produced by each ELISA. Table shows the ORs for GBC by antibody titres, using the quartiles of the control distribution to divide the subjects into four groups. A strong, highly statistically significant but inverse dose-response effect was observed in quartile analysis **[Table 3.5].**

Table 3.2 *H. Pylori* reactivity among study participant

H. pylori reactivity	GBC (n= 833)		Control (n=818)	
	Men(n=258)	Women(n=575)	Men(n=258)	Women(n=560)
***H. pylori* Sero-negative/non-reactive**	49(18.99)	112(19.48)	65(25.19)	118(21.07)
***H. pylori* Sero-positive/reactive**	209(81.01)	463(80.52)	193(74.81)	442(78.93)

Table 3.3 Percent Positive *H. pylori* reactivity, stratified by age-group

Age-groups	GBC (n= 833)		Control (n=818)	
	Men(n=258)	Women(n=575)	Men(n=258)	Women(n=560)
20-29	8(2.10%)	7(0.88%)	7(0.87%)	7(0.40%)
30-29	32(8.42%)	57(7.21%)	21(2.62%)	63(3.6%)
40-49	52(13%)	144(18.22%)	49(6.13%)	172(9.96%)
50-59	63(16.57%)	166(21.01%)	67(8.38%)	135(7.81%)
60+	54(14.21%)	89(11.26%)	49(6.13%)	65(3.76%)

Table 3.4 Percent Positive *H. pylori* reactivity, stratified by residential status

Current residential region	GBC (n= 833)		Control (n=818)	
	Men(n=258)	Women(n=575)	Men(n=258)	Women(n=560)
North	80(78.53%)	230(83.63%)	71(72.44%)	163(76.52%)
South	1(100%)	0(0%)	0(0%)	2(100%)
West	31(83.78%)	65(82.27%)	28(77.77%)	87(78.35%)
North-East	87(82.85%)	138(77.09%)	88(75.47%)	159(83.68%)
Central	10(76.92%)	30(71.42%)	14(82.35%)	31(70.45%)

Table 3.5 Odds ratio and 95% confidence intervals for GBC in relation to H. pylori IgG antibody

H. pylori reactivity	All (Cases: 833 , Controls: 818)					Men(Case: 258 , Controls: 258)					Women(Cases: 575 , Controls : 560)				
	Cases|Controls	OR (95%CI)[a]	P value[a]	OR (95%CI)[b]	P value[b]	Cases|Controls	OR (95%CI)[c]	P value[c]	OR (95%CI)[d]	P value[d]	Cases|Controls	OR (95%CI)[c]	P value[c]	OR (95%CI)[e]	P value[e]
<20	161|183	**Reference**		**Reference**		49|65	**Reference**		**Reference**		112|118	**Reference**		**Reference**	
≥ 20	672|635	1.13 (0.88-1.45)	0.308	1.07 (0.81-1.43)	0.602	209|193	1.39 (0.91-2.12)	0.126	1.34 (0.81-2.21)	0.247	463|442	1.02 (0.76-1.39)	0.852	0.95 (0.66-1.37)	0.824
Antibody Tertiles															
10-15 U/ml	106|138	**Reference**		**Reference**		**29|52**	**Reference**		**Reference**		**77|86**	**Reference**		**Reference**	
16-75 U/ml	342|268	1.63 (1.20-2.22)	0.002	1.71 (1.19-2.46)	0.003	109|77	2.47 (1.43-4.28)	0.001	2.56 (1.32-4.96)	0.005	233|191	1.36 (0.93-1.98)	0.108	1.41 (0.89-2.22)	0.134
76-988 U/ml	385|412	1.13 (0.84-1.53)	0.387	1.14 (0.80-1.61)	0.457	120|129	1.65 (0.97-2.78)	0.06	1.81 (0.96-3.42)	0.064	265|283	0.95 (0.66-1.37)	0.808	0.87 (0.56-1.36)	0.557
P trend			0.626		0.572			0.238		0.182			0.413		0.311

Abbreviations: CI, Confidence Interval; N, Number; OR, Odds ratio

[a]Adjusted for age(continuous), gender and education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[b]Adjusted for age(continuous), gender, education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking (yes/no)

[c]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south)

[d]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking (yes/no)

[e]Adjusted for age(continuous), education(Less than 5 years of schooling, ≥5 year of education), current residential region(north, north-east, central, west, south), waist to hip ratio (continuous), gallstone history (present/not present) , tobacco chewing and tobacco smoking(yes/no), total number of full term pregnancies(continuous)

3.8 Discussion

H. pylori has been classified as a type I carcinogen by International Agency for Research on Cancer (IARC). A causal association between chronic gastric infection *H. pylori* and development of gastric adenocarcinomas is well established(48). Currently, multiple human clinical studies have associated *Helicobacter* species particularly with *H. pylori* has been suggested to infect biliary tract and cause cancer. Although, *H. pylori* have been detected in human bile, whether this organism rarely colonizes the bile duct or gallbladder is not clearly documented.

Although the hypothesis regarding role of *H. pylori* in GBC was based on small scale observational studies, animal experiments have suggested possible mechanism by which *H. pylori* could cause GBC. We could not demonstrate statistically significant association in the present study. However, we observed borderline association in men, and some evidence of increased risk in one of the categories (e.g. 16-75 U/ml). It is therefore that despite of overall negative association observed in our study, we could not nullify underlying hypothesis about *H.pylori* and GBC. This observed borderline association could be due constraints in case-control study design and methodology used for detection of *H. Pylori* infection (i.e. *H. Pylori* IgG antibody), it would be prudent to detect *H. pylori* in gallbladder tissue or in bile for confirmation of finding. It would be prudent to detect *H. pylori* in gallbladder tissue or in bile for confirmation of finding as serological markers are not sensitive. It is also possible that co-infection of *H. pylori* an *H. bilis* might have modulated the infection with *H. pylori*. As *H. bilis* can survive in alkaline pH, few small studies have suggested role of *H.bilis* in GBC aetiology (50). However, we have not estimated infection with *H.bilis* in current study because of non availability of serological test which is sensitive and specific for detection of *H. bilis*.

Study provided an opportunity to observe the prevalence of *H. pylori* infection in different regions of India as control participants in the current study were enrolled from different regions. *H. Pylori* seroprevalence was observed to be 80%. No geographical diversity was noted in seroprevalence across different geographical regions of India.

In conclusion, study did not observe association of GBC with *H. pylori*, however we cannot rule out the role of *H. pylori* infection in etiopathogenesis of gallbladder cancer. In future, development of antibody assay targeting specific individual *Helicobacter* species and validation in tumour tissue or in bile will be able to confirm study findings.

3.9 Summary

H. pylori prevalence is high in all regions of India (75-81%). Seroprevalence does not show geographical variation. Seroprevalence increases with increase in age. Seroprevalence was prominent in both GBC cases and controls. Present study did not observe a significant association between *H.Pylori* infection and GBC, thus further studies are needed to confirm finding.

Chapter 4
Conclusion and future perspective

4.1 Conclusions

The GBC is one of the most common cancers in the North and North-east India. The disease is highly lethal and 5 years survival rates were reported to be 5%. Currently no effective treatments are available to treat GBC. There are no large scale studies globally or within India to understand the aetiology of GBC. The current study was therefore undertaken to understand the role of lifestyle factors and *H. pylori* infection in development of GBC. The major highlights of the work are as follows **[Table 4.1].**

1. The observation that the place of birth is an important risk factor and that increased risk persisted even after migration from high risk to low risk regions, indicating the probable role of both gene and environment. Thus, large scale genetic studies required to understand the role of genetic factors and their interaction with environmental factors in development of GBC.
2. Gallstone history is a predominant risk factor for GBC.
3. This is the first study globally to demonstrate the role of bidi smoking and tobacco chewing in development of GBC. The attributable risk for developing GBC was estimated as 8% for bidi smoking and 11% for tobacco chewing.
4. Following findings of the study should be disseminated and confirmed in future studies.
    i. Future studies should investigate if the increased risk with mustard oil consumption is because of some unmeasured confounding or because of adulteration of mustard oil or there is any other mechanism involved.
    ii. High consumption of fresh fish is observed to be risk for GBC. Future studies are needed to replicate this finding, exploring the role of possible infectious

agents, such as *Opisthorchis viverrini*, *Opisthorchis, felineus*, and *Clonorchis sinensis* or other mechanisms which may explain this finding.

iii. Central obesity is observed to increase GBC risk. Public health strategies should be developed for reducing central obesity, as it is not an only risk factor GBC but also for many other types of cancers and non communicable disease.

iv. The more the number of full term pregnancies (≥4) , higher the risk of GBC

As there is currently no effective treatment available for GBC, it is urgently required to confirm association identified in study regarding reduced consumption of tobacco, mustard oil and fresh fish. Results also indicate that central obesity is a strong risk factor for GBC. Controlling central obesity before it reaches epidemic proportion could mitigate risk for not only GBC, but also for many other non-communicable diseases, and should be a public health priority.

Table 4.1 Summary of risk /protective factors of GBC acquired from current case control study

Risk Factors	Variable	Effect Direction (Present study)	Effect Direction (Previous studies)
Association possibly causal			
Place of Birth	Place of birth in GBC high risk region	↑↑	No data
Gallstone	Gallstone History (Present/not present)	↑↑	↑↑
Tobacco habits	Bidi smoking	↑↑	Limited data
	Tobacco chewing	↑↑	Limited data
Central Obesity	High waist circumference	↑↑	↑↑
	High waist to hip ratio	↑↑	↑↑
Reproductive and hormonal factors	High parity and high number of pregnancies	↑↑	↑↑
	Late menarche	↑	↑
	Duration since last birth	↑↑	No data
Dietary intakes	Mustard oil	↑↑	No data
Association Difficult to interpret			
Dietary intake	High consumption of fruits and vegetables	↓↓	↓↓
	High consumption of cereals and pulses	↓↓	Limited data
	High consumption of fenugreek	↑↑	No data
	High consumption of fresh fish	↑↑	No data
Infection	*Helicobacter pylori*	-	Not conclusive
Central Obesity	Higher body Size at age 10, 20	↑↑	No data
No association			
Dietary intakes	Any spice consumption	–	No data
	Any meat consumption	–	Limited data
	Dairy products consumption	–	Limited data
Reproductive and hormonal factors	Late first birth	–	↓
	Age at menopause	–	Not conclusive
	Spontaneous /induced abortion	–	No data
	Oral contraceptive use and duration of use	–	–

↑↑ : Moderate to large extent risk , ↓↓ : Moderate to large decrease in risk , ↑ : Slight increase in risk , ↓ : Slight decrease in risk , - : No association

4.2 Future perspective:

The current study identified the following lifestyle related variables which requires confirmation and possible public health action to reduce the burden of GBC:

a. Tobacco in the form of smoking and smokeless
b. Central obesity
c. High consumption of mustard oil
d. High consumption of fresh fish
e. High consumption of mustard oil
f. Low consumption of fruits and vegetables
g. Pregnancies more than or equal to 4

The infectious aetiology of GBC should be further investigated, particularly in the view of observed borderline association in men, and some evidence of increased risk in one of the categories of *H. pylori* antibody (e.g. 16-75 U/ml). Role of *H.Pylori* infection in aetiology of GBC requires evaluation in studies in which *H.Pylori* antibodies can be measure well before the onset of GBC, for reliable calculation of the true seroprevalence *H. pylori* in cases and controls. This will help to establish whether there could be a causal association between *H. pylori* infection and GBC. Detection of *H. pylori* in gallbladder tissue or in bile for confirmation of finding could be prudent to understand association between *H.pylori* infection and *GBC.*

Given the role of tobacco consumption and central obesity in relation to GBC as observed in present study and their association with many other cancer sites, stringent public health measures should be undertaken to reduce central obesity as well as tobacco consumption. Government of India has already undertaken various initiatives for tobacco control under national tobacco control programme, with India is among the first few countries to ratify The

World Health Organization Framework Convention on Tobacco Control (WHOFCTC). These policies were implemented with different level of success in different states of India. The strict implementation of theses polices at national level will help in reducing burden of GBC in addition to other non-communicable diseases.

Mustard oil and fresh fish were associated with increased risk of GBC in the present study. Given the strong potential of these factors to reduce the burden of GBC, an urgent replication of findings should be consider in other settings, followed by public health interventions if findings are replicated.

In view of the strong risk observed for individuals born in particular regions, and consistent increase in risk of GBC even after migration, large scale genetic studies (e.g. GWAS) can help to elucidate the underlying role of genetics in the aetiology of GBC.

Future studies should undertake more detailed imaging procedures to detect gallstone disease among controls so as to comparably measure the history of gallstone among cases and controls.

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Original Article

Place of birth and risk of gallbladder cancer in India

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Abstract

CONTEXT: Within India, the incidence of gallbladder cancer (GBC) is characterized by marked geographical variation; however, the reasons for these differences are unclear. **AIMS:** To evaluate the role of place of birth, length of residence, and effect of migration from high- to low-risk region on GBC development. **SETTINGS AND DESIGN:** Population-based cancer registries (PBCRs); case–control study. **SUBJECTS AND METHODS:** Data of PBCRs were used to demonstrate geographical variation in GBC incidence rates. A case–control study data examined the role of birth place, residence length, and effect of migration in etiology of GBC. **STATISTICAL ANALYSIS:** Rate ratios for different PBCRs were estimated using Chennai Cancer Registry as the reference population. Odds ratios (ORs) for developing GBC in a high-risk region compared to a low-risk region and associated 95% confidence interval (CI) were estimated through unconditional logistic regression models using case–control study. **RESULTS:** GBC shows marked variation in incidence with risk highest in Northeast regions and lowest in South India. OR of 4.82 (95% CI: 3.87–5.99) was observed for developing GBC for individuals born in a high-risk region compared to those born in a low-risk region after adjusting for confounders. A dose–response relationship with increased risk with increased length of residence in a high-risk region was observed (OR lifetime 5.58 [95% CI: 4.42–7.05]; $P_{trend} \leq 0.001$). The risk persisted even if study participant migrated from high- to low-risk region (OR = 1.36; 95% CI: 1.02–1.82). **CONCLUSIONS:** The present study signifies the importance of place of birth, length of stay, and effect of migration from high- to low-risk region in the development of GBC. The data indicate role of environmental and genetic factors in etiology of disease.

Key Words: Birth region, gallbladder cancer, population-based cancer registries

Introduction

Gallbladder cancer (GBC), although generally a rare malignancy, has marked geographical variation both globally and within the Indian subcontinent.[1,2] The current study was planned to assess the risk of GBC across different regions of India and to evaluate the role of birthplace, length of residence in high-risk region, and effect of migration from high to low-risk region in the development of GBC.

Subjects and Methods

Data extraction from population-based cancer registries

We used data from 23 population-based cancer registries (PBCRs)[3] to quantify geographical variation in GBC incidence within India. PBCRs were assigned to one of the five different geographical zones based on their location in India, i.e. North, Northeast, Central, West, and South.

Case–control study

We conducted a hospital-based case–control study of GBC at Tata Memorial Hospital (TMH), Mumbai, from September 2010 to June 2015. The study was approved by the TMH Institutional Review Board. We enrolled 1170 GBC cases and 2525 controls during this period. Cases were histopathologically/cytologically confirmed men or women with primary GBC (International Classification of Diseases for Oncology Version 3 site code C24) visiting TMH for diagnosis and/or treatment. Controls enrolled in the study were visitors to patients in TMH with cancers other than biliary tract cancer. The proportion of controls from visitors for any individual cancer site was not more than 20%, with the majority of controls being friends, neighbors, colleagues, in-laws, and spouses. Controls were frequency matched to cases on age (±10 years), gender, and region of residence at the time of enrolment. For regional matching, India was divided into five regions which are as follows: North (Uttar Pradesh, Bihar, Delhi, Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Chandigarh, and Uttarakhand), Northeast (Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Tripura, West Bengal, Jharkhand, and Orissa), West (Goa, Gujarat, Daman and Diu, Dadra and Nagar Haveli, Maharashtra), South (Andhra Pradesh, Karnataka, Kerala, Lakshadweep, Andaman and Nicobar, Tamil Nadu, Telangana), and Central (Madhya Pradesh and Chhattisgarh).

Exposure assessment

Definition of high- and low-risk regions

To quantify the geographical variation in GBC incidence, we divided Indian states and territories into high- and low-risk regions using incidence rates extracted from PBCRs. States were considered to be in high-risk region if PBCR existing in the state observed average age-adjusted rates of >5.0 per 100,000 persons.[3] All other PBCR states were considered as low-risk regions. In states with no cancer registry, we used the microscopic age-adjusted incident rate provided by the atlas of cancer in India[3] to classify the states into high- and low-risk regions using the same threshold of incidence rate. Thus, Bihar, Delhi, Himachal Pradesh, Punjab, Chandigarh, Rajasthan, Uttarakhand, Uttar Pradesh, Assam, Tripura, Sikkim, Jharkhand, and West Bengal were

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categorized into high-risk regions (mainly North Indian states) and the remaining states classified as low-risk regions.

Other exposure variables

We used questionnaire data from the case–control study to adjust for potential confounders. The questionnaire for case–control study included detailed information on lifestyle risk factors such as diet, tobacco and alcohol usage, gallstone history, and reproductive history. In addition, detailed anthropometric measurements were recorded for all study participants. All anthropometric measurements were done twice in succession and averaged for the final value.

Statistics

Detailed statistical analysis was performed on both cancer registry and case–control data. Analyses were performed using the Statistical Package Stata SE 12 (StataCorp LP, College Station, TX, USA).

Cancer registry data

We extracted gender-specific age-standardized incidence rates per 100,000 person-years for 23 different PBCRs included in the National Cancer Registry Programme. The Chennai Cancer Registry is high quality and of longest duration; registry with one of the lowest rates of GBC was used as reference category to estimate rate ratios (RRs) for each registry.[3] RRs for each registry were estimated.[4]

Case–control study data

Odds ratios (ORs) and their associated 95% confidence intervals (CIs) for developing GBC in high-risk region compared to low-risk region were estimated through unconditional logistic regression models using hospital-based case–control study. To estimate the effect of length of residence in a high-risk region, we stratified study participants from the hospital-based case–control study into the following mutually exclusive categories:

- Never lived in a GBC high-risk region (reference)
- Lived for a minimum of 1 year but <20 years in a GBC high-risk region
- Lived for 20 and more than 20 years but less than a lifetime in a GBC high-risk region
- Lifetime: If study participant has lived the entire course of his/her life in a GBC high-risk region.

Effect of migration on GBC risk was estimated by comparing the study participants born in high-risk region and then migrated to low-risk region at any point in their life course to those are not migrated in their entire life course.

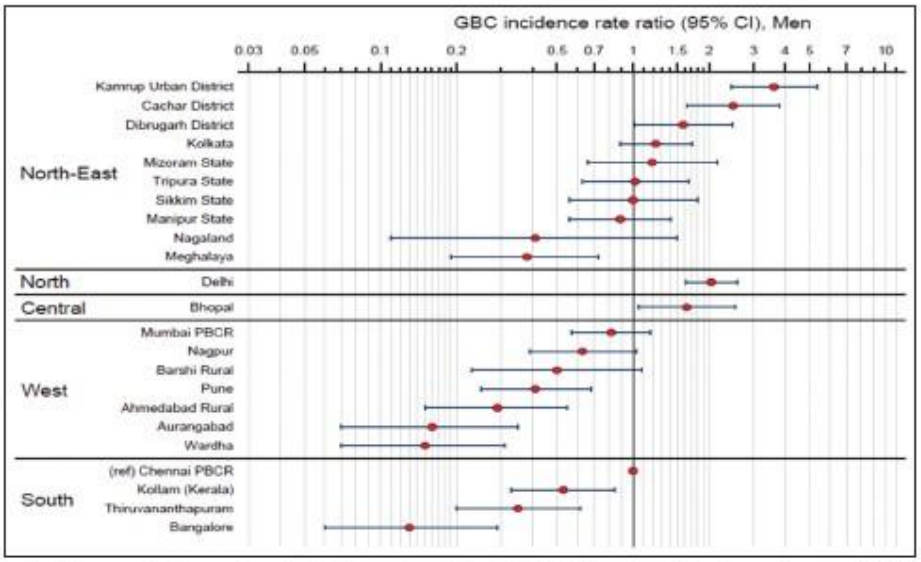
Models were adjusted for potential confounders; age (continuous), education (<5 years of schooling, ≥5 years of education), current residential region (North, Northeast, Central, West, and South), gender, gallstone history (present/not present), waist to hip ratio (continuous), smoking and smokeless tobacco usage (yes/no), and number of full-term pregnancies as continuous (women).

Results

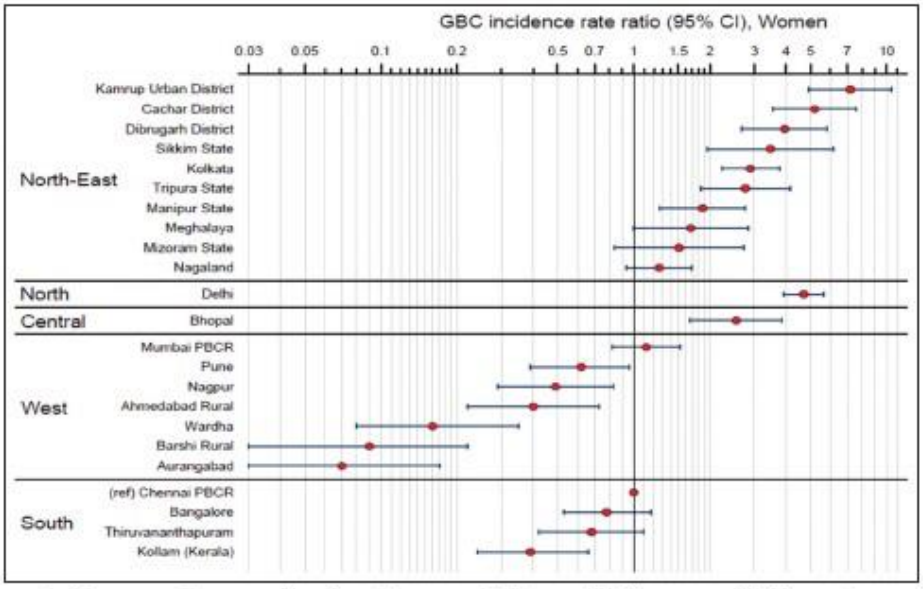
GBC incidence showed geographical variation in India [Graphs 1 and 2]. Kamrup Registry in the Northeast zone of India showed the highest RR (men: 3.61 and women: 7.18) compared to the the Chennai Cancer Registry in Southern India. The RR for registries in North and Northeast varied from 0.38–3.61 for men to 1.26–7.18 from women while registries from South showed lowest risk [Graphs 1 and 2].

The results of case–control study showed risk in both high- and low-risk regions [Tables 1 and 2]. Table 1 shows demographic information for participants enrolled in the hospital-based case–control study. The mean age for cases and controls was observed to be 49.85 and 46.03 years, respectively. Cases more often reported current residence in the North and Northeast region, with the smallest proportion of cases residing in South India.

The OR of developing GBC for those reporting birth in a high-risk region was observed to be 4.82 (95% CI: 3.87–5.99) compared to those born in low-risk



Graph 1: Gallbladder cancer incidence rates in men for selected cancer registries stratified by geographical zone. Source: NCRP-ICMR - Three years report of population-based cancer registries 2009–2011; Bangalore, 2013



Graph 2: Gallbladder cancer incidence rates in women for selected cancer registries stratified by geographical zone. Source: NCRP-ICMR - Three-year report of population-based cancer registries 2009–2011; Bangalore, 2013

Table 1: Selected characteristics of study participants by case–control status

Variables	Case, *n* (%)*, (*n*=1170)	Control, *n* (%)*, (*n*=2525)
Gender		
Male	380 (32.48)	799 (31.64)
Female	790 (67.52)	1,726 (68.36)
Age at enrolment (years)		
20-29	22 (1.88)	131 (5.19)
30-39	156 (13.33)	549 (21.74)
40-49	347 (29.66)	815 (32.28)
50-59	401 (34.27)	700 (27.72)
60 and above	244 (20.85)	330 (13.07)
Mean age (±SD)	49.85 (10.21)	46.03 (10.70)
Residential region at enrolment†		
North	534 (45.64)	619 (24.51)
South	3 (0.26)	34 (1.35)
West	156 (13.33)	1,086 (43.01)
Northeast	395 (33.76)	632 (25.03)
Central	82 (7.01)	154 (6.10)
Education at enrolment		
Illiterate	313 (27.38)	239 (9.48)
Literate	54 (4.64)	114 (4.52)
<5 years of schooling	88 (7.55)	151 (5.99)
5-8 years of schooling	260 (22.32)	564 (22.38)
High school	257 (22.06)	749 (29.72)
College graduation and more	187 (16.05)	703 (27.90)

*Percentage based on nonmissing values; †North (Uttar Pradesh, Bihar, Delhi, Haryana, Himachal Pradesh, Jammu and Kashmir, Punjab, Rajasthan, Chandigarh, and Uttarakhand), Northeast (Arunachal Pradesh, Assam, Meghalaya, Nagaland, Manipur, Tripura, West Bengal, Jharkhand, and Orissa), West (Goa, Gujarat, Daman and Diu, Dadra and Nagar Haveli, Maharashtra), South (Andhra Pradesh, Karnataka, Kerala, Lakshadweep, Andaman and Nicobar ,Tamil Nadu, Telangana), and Central (Madhya Pradesh and Chhattisgarh); ‡High- and low-risk GBC regions based on incidence data from the National Cancer Registry Programme and Cancer Atlas of India: Bihar, Delhi, Himachal Pradesh, Punjab, Chandigarh, Rajasthan, Uttarakhand, Uttar Pradesh, Assam, Tripura, Sikkim, Jharkhand and West Bengal were classified as high-risk regions, the remaining states and territories of India were classified as low-risk regions. SD=Standard deviation; GBC=Gallbladder cancer

region, after adjusting for potential confounders. The observed risk was much stronger in women (OR = 6.04; 95% CI: 4.52–8.07) compared to men (OR = 3.17; 95% CI: 2.23–4.50). Risk increased with increasing duration of residence in a high-risk region. The risk was maximum for those who always/lifetime lived in a high-risk region compared to those who never lived in high-risk region (OR = 5.58; 95% CI: 4.42–7.05). Study participants born in high-risk region reported high susceptibility of GBC even after migration from high- to low-risk region (OR = 1.36; 95% CI: 1.02–1.82) [Table 2].

Discussion

The current study using data from cancer registries demonstrates marked geographical variation in GBC risk, with the highest risks observed in registries from North and Northeast regions compared to the registries in South India. These results are based on 23 well functional PBCRs data. Some of these registries have also met the criteria for inclusion in a recent volume of cancer incidence in five continents.

To evaluate further whether the higher risk in particular region is because of genetic or environmental factors, we analyzed data from the ongoing case–control study at TMH using information on birthplace and residential histories obtained by personal interviews by trained staff. We observed higher risk of developing GBC if birthplace was in high-risk region compared to that in low-risk region and the risk increased with increase in duration of residence in high-risk region. The increased risk persisted but reduced if individuals migrate from high- to low-risk region indicating either continuing behavioral differences in these individuals or the role of hereditary factors in development of GBC. A large proportion of GBC cases currently residing in low-risk regions were born in a high-risk region (44.81%),

Table 2: Residence in high-risk region and risk of developing estimates from case-control study in Mumbai (2010–2015)

Parameters	All (case: 1170, control: 2525)			Men (case: 380, control: 799)			Women (case: 790, control: 1726)		
	n (case|control)*	OR (95%CI)†	OR (95%CI)‡	*n* (case|control)*	OR (95%CI)†	OR (95%CI)§	*n* (case|control)*	OR (95%CI)†	OR (95%CI)‖
Birth region									
Low-risk region	189|1274	Reference		69|329	Reference		120|945	Reference	
High-risk region	979|1250	5.76 (4.77-6.95)	4.82 (3.87-5.99)	311|469	3.62 (2.64-4.96)	3.17 (2.23-4.50)	668|781	7.15 (5.65-9.06)	6.04 (4.52-8.07)
Length of residence in high-risk region									
Never lived	169|1216	Reference		66|317	Reference		103|899	Reference	
Ever lived	995|1302	6.14 (5.04-7.46)	5.09 (4.06-6.38)	313|479	3.59 (2.61-4.94)	3.15 (2.20-4.49)	682|823	8.00 (6.24-10.27)	6.72 (4.95-9.12)
<20 years	46|92	4.01 (2.67-6.01)	3.04 (1.85-4.97)	15|30	2.83 (1.42-5.62)	3.16 (1.51-6.62)	31|62	4.66 (2.81-7.72)	2.49 (1.25-4.95)
≥20 years and less than lifetime	97|171	4.12 (3.02-5.62)	4.02 (2.82-5.73)	39|67	2.86 (1.76-4.64)	2.88 (1.69-4.91)	58|104	5.03 (3.36-7.53)	5.43 (3.33-8.86)
Lifetime	852|1039	6.85 (5.59-8.38)	5.58 (4.42-7.05)	259|382	3.84 (2.76-5.35)	3.21 (2.21-4.66)	593|657	9.12 (7.05-11.80)	7.65 (5.58-10.49)
P trend test		≤0.001	≤0.001		≤0.001	≤0.001		≤0.001	≤0.001
Migration from high- to low-risk region									
No	1045|2318	Reference		328|715	Reference		717|1603	Reference	
Yes	123|206	1.28 (1.00-1.64)	1.36 (1.02-1.82)	52|83	1.34 (0.92-1.95)	1.63 (1.08-2.46)	71|123	1.27 (0.92-1.76)	1.21 (0.80-1.84)

*Cases and controls; †Adjusted for age (continuous), gender and education (<5 years of schooling, ≥5 year of education), current residential region; ‡Adjusted for age (continuous), gender, education (<5 years of schooling, ≥5 year of education), current residential region, waist to hip ratio (continuous), gallstone history (present/not present), tobacco chewing and smoking habits (yes/no); §Adjusted for age (continuous), education (<5 years of schooling, ≥5 year of education), current residential region, waist to hip ratio (continuous), gallstone history (present/not present), tobacco chewing and smoking habits (yes/no); ‖Adjusted for age (continuous), education (<5 years of schooling, ≥5 year of education), current residential region, waist to hip ratio (continuous), gallstone history (present/not present), tobacco chewing and smoking habits (yes/no), no of full-term pregnancies (continuous). CI=Confidence interval, OR=Odds ratio

suggesting that moderately higher rates in some of low-risk regions (such as Mumbai) might partially be due to migration of population from high-risk regions. The prevalence of possible risk factors[5] such as gallstones,[6] *Salmonella typhi*,[7] obesity,[8] and heavy metals[9] was observed to be high in high-risk region of GBC (particularly, in North and Northeast regions). It can be thus speculated that differences in the risk of GBC are partly explained by differences in these potential risk factors.

Our study is the first to demonstrate the role of birth place and effect of migration in development of GBC. These findings are based on well-conducted case–control study with stringent quality control measures and after carefully adjusting for potential confounders.

The findings of current study demonstrate need for careful evaluation of possible environmental and genetic factors to estimate the risk attributable to environmental and genetic factors in the development of GBC.

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Conflicts of interest

There are no conflicts of interest.

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