BANARAS HINDU UNIVERSITY
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No. Dean/2022/EC/3226

Dated: 28.02.2022
25.03

Dr. Jyotsna Kailashiya
Associate Professor
Department of Biochemistry
Institute of Medical Sciences
Banaras Hindu University

Dear Madam,

The Ethics Committee meeting was held on 28.02.2022 at 2:30 PM in the Chamber of the Dean, Faculty of Medicine, IMS for Ethical clearance of the MD/MS/DM/M.Ch/Ph.D/synopsis/Projects submitted by the following:

Name of the PI	Dr. Jyotsna Kailashiya
Study Title	Development of method for screening and early detection of gall bladder cancer
Suggestions/Comments	-
Remarks	**The Study is approved by the Institutional Ethics Committee**

This is for your information and necessary action at your end.

(DR. KIRAN GIRI)
MEMBER SECRETARY

Yours sincerely,

V. Bhattacharya

(PROF. V. BHATTACHARYA)
CHAIRPERSON OF THE ETHICAL COMMITTEE

Revised summary of project- for ethical approval

Project title: ***Development of methods for screening and early detection of gall bladder cancer.***

Investigators:

1. Dr. Jyotsna Kailashiya
   Department of Biochemistry, IMS, BHU, Varanasi.

2. Dr. Shashi Prakash Mishra
   Department of Surgery, SSH Hospital, IMS, BHU, Varanasi

3. Dr. Ashish Verma
   Department of Radio-diagnosis, SSH Hospital, IMS, BHU, Varanasi.

4. Dr. Vikas Kailashiya
   Department of Pathology, IMS, BHU, Varanasi.

5. Dr. Soumen Kanti Manna
   Saha Institute of Nuclear Physics, Kolkata.
   (Consent attached)

Background: Gallbladder cancer (GBC) shows a peculiar geographical distribution with high prevalence in few Latin American, East Asian countries and Indian subcontinent. The incidence rate is particularly high in Gangetic belt and north-east India, including Varanasi, UP (Dutta, Bush et al. 2019). Women are 3-4 times more likely to have the disease compared to men. Some mutations and over expression have also been found to be linked with GBC gene (Nigam, Misra et al. 2010, Singh, Mishra et al. 2016). It is one of the worst cancers with 5-year survival rate <10% in patients with involvement of lymph node or other organs. The median survival has also not improved much in decades and hovers around six months post-diagnosis. Research effort in developed world remains limited due to low incidence. Lack of early diagnosis worsens patient outcome.

Challenges:

- Silent disease progression with several clinical presentations overlapping with other hepatobiliary pathologies.
- While surgery is the main-stay of treatment, around 80% patients present as inoperable disease because of late diagnosis.
- Treatment options are limited and outcomes are poor.

Often, GBC is incidentally detected in gallstone (GS) patients. Thus, clinicians often recommend radical removal of the gallbladder to gallstone patients. This is a double edged sword. On one hand, only a fraction of GS eventually progress to GBC leading to unnccessary removal of the organ, which

comes with it's own physiological complication apart from aesthetic and psychological issues. On the other hand, people not opting for GB removal run the risk of developing GBC. Thus, development of a method for minimally invasive screening and monitoring for GBC in GS patients can significantly help patients and clinicians. In a nutshell, early biomarker specific to GBC is highly warranted. This may help to monitor patients at risk of GBC as well as monitoring of therapeutic response. Given that metabolic reprogramming is a hallmark of neoplastic transformation and derangement of cholesterol homeostasis plays a central role in development of gallstones, lipidomic and metabolomic analysis of biofluids hold the promise of yielding such biomarkers.

Objectives:

Objective 1: Detailed demographic, anthropometric, clinical and biochemical characterization of gallstone patients, with suspected gall bladder cancer, undergoing radical surgery.

Objective 2: Metabolomic and lipidomic analysis of serum, urine, and saliva samples towards identification of minimally invasive signatures associated with gallbladder cancer in gallstone patients.

Objective 3: Analysis of mechanistic association between putative minimally invasive biomarkers and underlying molecular pathways associated with gallbladder cancer.

Materials and Methods:

Study type: Observational, cross-sectional

Study population: Patients (18-65 years) reporting to SSH, IMS-BHU with symptoms and USG impression suggestive of gallstone (GS) disease or gallbladder cancer (GBC) and consenting for radical surgical removal of gall bladder.

Table 1: Exclusion and inclusion criteria for recruitment of gallstone/GBC patients

Inclusion criteria	Exclusion criteria
-USG impression suggestive of GS and/or GBC -Consenting to radical surgery	-Age > 65 yr, <18 yr -Not consenting to radical surgery -Already undergone GB surgery/removal -Pregnancy -Other malignancy or terminal illness -Undergone any other major surgery, requiring anesthesia within 1 month -COVID positive within 1 month

Sample size: 80-100 subjects each in gall bladder cancer, gall stone (no cancer) category and 30 healthy controls.

Sample size calculation:

Prevalence: A preliminary observation and previous reports (Singh, Ansari et al. 2012, Dutta, Bush et al. 2019) suggests that incidence of GBC among GS patients reporting to IMS-BHU surgery, is approximately 5%. Thus, the sample size for ascertaining prevalence may be calculated using the following formulae.

$$n = \frac{z^2\,\hat{p}(1-\hat{p})}{\varepsilon^2}$$

With a margin of error (ε) = 0.05 at 95% confidence interval (z = 1.96) and estimated 5% GS cases requiring radical surgery (for GBC), the total number of GS patients enrolled in the study should be **>73**. However, in order to find meaningful biomarkers to stratify a fresh GS case as GS or GBC, the sample size per group needs to be much higher as mentioned below.

Biomarker discovery cohort: For a 1.5-fold change in mean metabolite/lipid concentration between GS and GBC with 80% power and alpha = 0.05 (after Bonferroni correction for multiple comparisons involving 500 metabolites/lipids) and 60% standard deviation, the sample size per group should be approximately 65. So, for discovery cohort, **70 subjects will be recruited per group** (GBC positive and negative). This would require initial screening of ~1400 subjects (with GS suspected in USG) to get the requisite number of incidental GBC cases. In order to speed up recruitment of GBC cases, those with GBC suspected in USG or other imaging/tests will also be included. Age, gender and BMI-matched GS cases will form the control group.

Biomarker validation cohort (test set): For a 1.5-fold change in mean metabolite/lipid concentration between GS and GBC with 80% power and alpha = 0.05 (after Bonferroni correction for multiple comparisons involving top 10 metabolite/lipids) and 50% standard deviation, the sample size per group should be approximately 27. So, for discovery cohort, minimum **30** subjects will be required per group.

Sampling Strategy:

Initial screening: Patients will be screened based on clinical suspicion of GS and/or GBC and USG report. Pre-operative samples from patients showing GS/GBC in USG and consenting to radical surgery/GB removal will be enrolled in this study after explanation of procedures and obtaining informed written consent (proforma attached). Participants will be categorised in GS (control) and GBC (cases) groups after post operative histopathology examination. Samples will also be collected after the surgery and before commencement of any treatment such as chemo- or radiotherapy from willing follow up patients. Comparative analysis of these samples would help to identify signatures specifically associated with presence of gallstone or gallbladder cancer. Samples will be divided into test and validation cohort to further check sensitivity and specificity of signatures of interest. In the

validation cohort, an additional set of age-, gender and BMI-matched controls with no indication of GS or GBC or any other gall bladder disease (GBD) upon USG will be recruited to check sensitivity and specificity of putative diagnostic signatures. The workflow for sample collection and analysis is shown in Figure 1A. Figure 1B depicts the rationale behind collection of both pre- and post-operative samples to tease out signatures not strictly associated with presence of GBC.

Work plan:

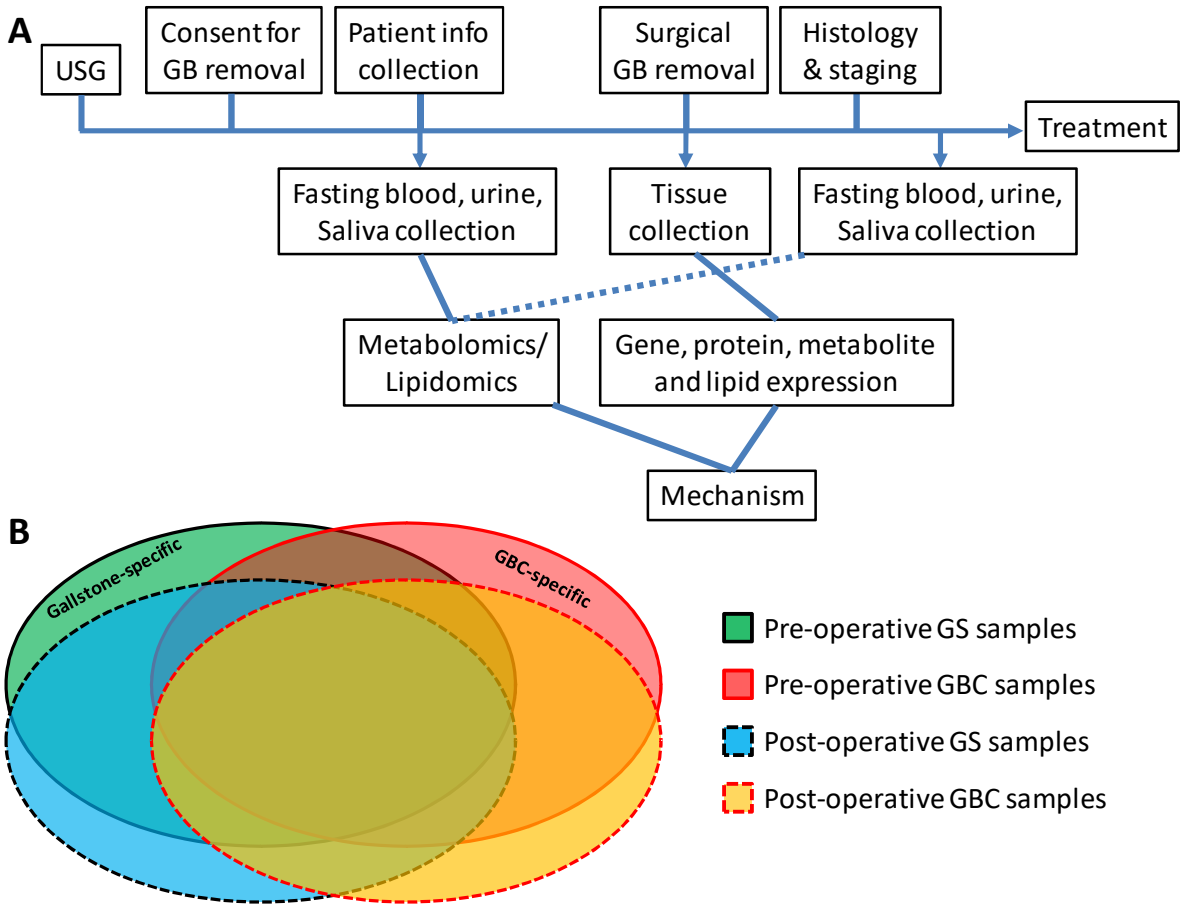

Figure 1: (A) Executive workflow of the study. (B) Schematic representation showing the rationale behind comparative metabolomic/lipidomic analysis of both pre- and post-operative samples from GS and GBC cases. This will help to identify GS-specific (green) and GBC-specific (pink) differential signatures that should either significantly diminish or cease to exist as differential signatures once the

Protocols for each objective:

Objective 1: Detailed demographic, anthropometric, clinical and biochemical characterization of gallstone patients undergoing radical surgery.

Anthropometric, epidemiological, clinical and radiological assessment: Body weight and height shall be recorded at the time of subject recruitment, before surgery. Age, habitat, socioeconomic, medical (self and family) history will be recorded as per patient information document appended below. Hematological, biochemical and radiological assessment (USG, CT, MRI or PET scan) will be performed as recommended by the clinician and consent of patients. Details of therapeutic interventions as per recommendation of the clinician will be recorded.

Routine Biochemical tests: Results of routine biochemical assays including serum urea, creatinine, liver function tests including LDH, bilirubin as well as fasting glucose and lipid profile analysis will be recorded from patients' hospital records as per availability.

Sample collection: A part of the fasting blood samples collected for routine hematological and biochemical assessments from consenting patients with suspected GS and/or GBC will be used for the study. Fasting urine samples will be collected from patients in 50 ml sterile polypropylene tubes on the same day. Patients will be asked not to ingest saliva for 5 min while sitting and spontaneous saliva will be collected in 5 ml sterile polypropylene tubes. Excised GB tissue will be sent for histology. A part of the excised tumor or normal (or adjacent normal in case of GBC) tissue will be washed with PBS and stored. Collection of fasting blood, urine and saliva will be repeated after arrival of biopsy report of the excised gallbladder tissue and before initiation of any treatment including radiotherapy or chemotherapy for GBC patients. Blood and urine samples from volunteer 30 healthy individuals (without GBC or GS) will also be collected for control group. All ethical guidelines and aseptic conditions will be maintained during sample collections.

Histolopathology: Histopathological examination will be performed to identify presence of GBC as per routine protocol using formalin-fixed paraffin embedded tissue section to assess the tumor grade.

Staging: Disease staging will be performed as per TNM staging protocol.

Objective 2: Metabolomic and lipidomic analysis of serum, urine, and saliva samples towards identification of minimally invasive signatures associated with GBC in GS patients.

Sample processing, storage and transport: Serum will be prepared as per routine protocol by centrifugation. Urine and saliva samples will be centrifuged at 20000g for 25mins at 4'C and

supernatants will be stored as 1ml and 0.5 ml aliquots, respectively for urine and saliva. These samples as well as urine will be stored at -80C until further processing. Tissue samples will also be stored at -80'C until further processing. De-identified/coded samples/extracts will be shipped to Dr. Soumen Kanti Manna at Saha Institute of Nuclear Physics, Kolkata in dry ice, maintaining proper cold chain. Containers will be thoroughly decontaminated upon arrival. All sample processing will be done with appropriate protective gears and inside fume hood.

Metabolite and lipid extraction: Serum/plasma samples will be extracted by biphasic solvent extraction method involving methanol/water/chloroform (modified Bligh-Dyer method) to harvest metabolites from the upper aqueous layer and lipids from the lower organic layer. Metabolites from urine and saliva will be extracted by monophasic solvent mixture comprising isopropanol, water and acetonitrile. Both methods would also denature all proteins and inactivate any pathogen.

Metabolomic and lipidomic analysis:

GC-MS: Extracted metabolites will be derivatized with MSTFA or ethylchloroformate to make them amenable to GC-MS analysis. For fatty acid analysis, Methanolic HCl will be used to produce fatty acid methyl esters (FAME). Compounds will be separated on a HP5-MS column and analyzed by single-quad MS.

LC-ESIMS: Lipid extract will be diluted in acetonitrile/isopropanol/water (1:2:1) and transferred to glass sample vials for ESIMS analysis. Metabolites will be diluted with 50% acetonitrile and used for ESI-MS analysis. Lipids and metabolites will be separated on a reverse phase (C18) column prior to introduction to ESIMS. Samples will be run in a randomized manner with intermittent injection of pooled and authentic standard mixture samples for quality control.

Data analysis: Chromatograms will be aligned, deconvoluted and binned to extract features. Features with CV> 20% in QC samples will be discarded. SIMCA-P+ (Umetrix, Umea, Sweden) and MetaboAnalyst (http://www.metaboanalyst.ca/) will be used for multivariate statistical analysis of metabolomic and lipidomic data matrix. Unsupervised (PCA) as well as supervised (PLS-DA and OPLS-DA) pattern recognition analysis will be performed to identify signatures contributes to separation of metabotypes associated with respective pathologies. List of differential features identified in pre-operative GS-vs-GBC cases will be compared with those identified in post-operative samples to select features strongly associated with presence of GS or GBC as shown in Figure 2B. The chemical identity of these signatures will be established using fragmentation analysis, database mining and authentic standards. Pathways involved will be analyzed using MetaboAnalyst or Mass Profiler Pro (Agilent, Santa Clara, USA) using FDR-corrected set of differentially abundant metabolites.

Integrated pattern recognition and model building: FDR correction will be used to screen features for subsequent analysis. Correlation between demographic, anthropometric, biochemical, metabolomic and lipidomic features will be analyzed. Intra-feature correlation will be examined to identify features with correlation coefficient >0.9. Random Forest analysis will be used to build models and screen features to distinguish GBC from GS. 20-fold leave-one-out validation and label swapping will be used to test robustness of model performances. Regression and receiver operating characteristic (ROC) analysis will be used to build models, test sensitivity and specificity of individual as well as panel of differentiating features. Among models with comparable performance, those involving most mechanistically connected features (see below) will be further chosen to analyze model performance in the test cohort. Further, differential metabolites/lipids that are identified to be strictly associated with GBC will be given preference while selecting the biomarker panel.

Objective 3: Analysis of mechanistic association between putative minimally invasive biomarkers and underlying molecular pathways associated with gallbladder cancer.

Metabolomic and lipidomic analysis of tissue: In order to examine if the derangement of metabolite and lipid composition in GBC is directly caused by changes in the tumor, comparison of metabolomic signatures of tumor and paired adjacent normal (from GBC) or normal (GS) tissues will be performed. Tissue samples will be homogenized in mixture of organic solvent as mentioned above to release metabolites, lipids and denature proteins. Upper aqueous and lower organic layers will be used for metabolomic and lipidomic analysis as indicated above. Correlation between changes in tumor metabolome and biofluid metabolome will be analyzed. Combined tissue and biofluid metabolome data will be subjected to metabolite set enrichment analysis using MetaboAnalyst to examine contribution of tumor metabolic reprogramming to changes in biofluid metabolome.

Pathway Analysis and mechanistic relationship: FDR corrected differential metabolomic and lipidomic signatures will be combined to analyze differentially regulated pathways in GBC compared to GS using Ingenuity Pathway Analysis or Cytoscape. Upstream regulators of these pathways will be identified through bioinformatics. Expression of genes associated with these pathways will be examined using RTPCR. Expression of proteins and their post-translational modifications will be examined by western blot using respective antibodies as applicable.

Genomics and transcriptomics analysis and RTPCR: Genomic DNA will extracted from whole blood by Tris HCl-Chroloform-alcohol precipitation method, for detection of mutations (eg p53) (Nigam, Misra et al. 2010, Singh, Mishra et al. 2016). RNA will be extracted from homogenized tissue using

Trizol reagent. cDNA preparation and RTPCR will be performed using commercially available kits with primers targeting genes of interest, for transcriptome analysis.

Protein extraction and western blot: Protein will be extracted from homogenized tissue using RIPA buffer. Western blot for analysis of protein expression and modification will be performed following standard protocol using commercially available antibodies against protein of interest.

Data Sharing and privacy: All patient samples, information, medical records, clinical, biochemical, hematological and radiological data will be coded and made anonymous. Only treating clinician shall have access to personally identifiable information which will be used strictly for treatment and follow-up. Anonymous patient data and de-identified samples will be shared with collaborators for research purposes only.

Ethical Considerations: Study will commence after receiving approval from Institutional Ethics Committee (IEC). The study will be conducted in conformity with all ethical guidelines. Only those persons willing to participate in the study and sign an informed consent form will be included. Identity of all the study subjects will be kept confidential.

Impact: This study will help to identify minimally invasive biomarker(s) and a method to identify GBC. This will help to screen and stratify gallstone patients certainly requiring radical surgery. Subject to further studies these may also be helpful in screening and monitoring for GBC development in patients not opting for surgery.

References:

Dutta, U., N. Bush, D. Kalsi, P. Popli and V. K. Kapoor (2019). "Epidemiology of gallbladder cancer in India." Chin Clin Oncol **8**(4): 33.

Nigam, P., U. Misra, T. Negi, B. Mittal and G. Choudhuri (2010). "Alterations of p53 gene in gallbladder cancer patients of North India." Tropical Gastroenterology **31**(2): 96-100.

Singh, A., P. K. Mishra, S. S. Saluja, M. A. Talikoti, P. Kirtani and A. K. Najmi (2016). "Prognostic Significance of HER-2 and p53 Expression in Gallbladder Carcinoma in North Indian Patients." Oncology **91**(6): 354-360.

Singh, S., M. A. Ansari and G. Narayan (2012). "Pathobiology of gall bladder cancer." J Sci Res **56**: 35-45.

Patient Information Sheet

Patient ID:

Gender: M ◯ **F** ◯ **Others** ◯

Age: (Years)

Height: (cm) **Body weight at Hospitalization:** (kg)

Village/town: **P.S.:** **District:**

Educational qualification:

Occupation:

Food habit: Jain ◯ Vegetarian ◯ Occasional eggetarian ◯ Regular eggetarian ◯ Occasional non-vegetarian ◯ Occasional non-vegetarian ◯

Did you heard of gallbladder cancer in the locality? Yes ◯ **No** ◯

Did you know that gallstone is a risk factor for gallbladder cancer? Yes ◯ **No** ◯

Intensity of upper abdominal pain: (scale of 0-10)

Duration of upper abdominal pain: (months/weeks/days)

Clinical Presentation before admission/surgery:

Upper abdominal pain ◯ Right upper abdominal pain ◯ Back pain ◯ Right shoulder pain ◯ Chest pain ◯ Jaundice ◯ Nausea ◯ Vomiting ◯ Loss of appetite ◯ Indigestion ◯ Diarrhoea ◯ Tiredness ◯ Loss of weight ◯ Change of taste ◯ Fever ◯ Chills ◯

Clinical history:

T2DM ◯ Cardiovascular disease ◯ Obesity ◯ Typhoid ◯ Paratyphoid ◯ Hypertension ◯ Hypotension ◯ Hypercholesterolemia ◯ Hyperlipidemia ◯ NAFLD ◯ NASH ◯ Viral hepatitis ◯ Jaundice ◯ Alcoholic liver disease ◯

Life-style history:

Regular smoker ◯ Occasional smoker ◯ Regular drinker ◯ Occasional drinker ◯

Ongoing medication(s):

Family history of gallstone:

Father ◯ Mother ◯ Brother ◯ Sister ◯ Paternal uncle ◯ Paternal aunt ◯ Maternal uncle ◯ Maternal aunt ◯ Paternal grandfather ◯ Paternal grandmother ◯ Maternal grandfather ◯ Maternal grandmother ◯

Family history of gallbladder cancer:

Father ◯ Mother ◯ Brother ◯ Sister ◯ Paternal uncle ◯ Paternal aunt ◯ Maternal uncle ◯ Maternal aunt ◯ Paternal grandfather ◯ Paternal grandmother ◯ Maternal grandfather ◯ Maternal grandmother ◯

Family history of liver cancer:

Father ◯ Mother ◯ Brother ◯ Sister ◯ Paternal uncle ◯ Paternal aunt ◯ Maternal uncle ◯ Maternal aunt ◯ Paternal grandfather ◯ Paternal grandmother ◯ Maternal grandfather ◯ Maternal grandmother ◯

Family history of other GI cancer:

Father ◯ Mother ◯ Brother ◯ Sister ◯ Paternal uncle ◯ Paternal aunt ◯ Maternal uncle ◯ Maternal aunt ◯ Paternal grandfather ◯ Paternal grandmother ◯ Maternal grandfather ◯ Maternal grandmother ◯

Family history of non-GI cancer:

Father ◯ Mother ◯ Brother ◯ Sister ◯ Paternal uncle ◯ Paternal aunt ◯ Maternal uncle ◯ Maternal aunt ◯ Paternal grandfather ◯ Paternal grandmother ◯ Maternal grandfather ◯ Maternal grandmother ◯

Family history of hypercholesterolemia/hyperlipidemia:

Father ◯ Mother ◯ Brother ◯ Sister ◯ Paternal uncle ◯ Paternal aunt ◯ Maternal uncle ◯ Maternal aunt ◯ Paternal grandfather ◯ Paternal grandmother ◯ Maternal grandfather ◯ Maternal grandmother ◯

Family history of obesity/T2DM/cardiovascular disease:

Father ◯ Mother ◯ Brother ◯ Sister ◯ Paternal uncle ◯ Paternal aunt ◯ Maternal uncle ◯ Maternal aunt ◯ Paternal grandfather ◯ Paternal grandmother ◯ Maternal grandfather ◯ Maternal grandmother ◯

USG impression:

Preoperative samples collected: Serum ◯ Urine ◯ Saliva ◯ **Date:**

Normal tissue: (Y/N) Tumor tissue: (Y/N) Tissue @-80C: (Y/N) Bile sample: (Y/N)

Biopsy impression:

Clinical/hematological/biochemical parameters:

Diagnosis and recommendation:

Post-operative samples collected: Serum ◯ Urine ◯ Saliva ◯ **Date:**

Authorized signatory