Excretion of heavy metals from the biliary tract and its association with pancreato-biliary malignancy

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CONTENTS

LIST OF FIGURES 4
LIST OF TABLES 6
ACKNOWLEDGEMENT 7

1. INTRODUCTION 13
MORPHOLOGY OF BILIARY AND PANCREATIC CANCER 13
PATHOLOGY OF BILIARY AND PANCREATIC CANCER 15
Cholangiocarcinoma 15
Gallbladder Cancer 17
Pancreatic and periampullary carcinoma 18
PANCREATO-BILIARY MALIGNANCY – PROGNOSIS & WORLDWIDE TRENDS IN MORTALITY 19
EPIDEMIOLOGY 23
Cholangiocarcinoma 23
Intra-hepatic cholangiocarcinoma 24
Extra-hepatic Cholangiocarcinoma & Gallbladder cancer 26
Pancreatic Cancer 27
RISK FACTORS FOR PANCREATO-BILIARY MALIGNANCY 28
Cholangiocarcinoma 28
Gallbladder carcinoma 33
Pancreatic & periampullary cancer 48
MOLECULAR PATHOGENESIS OF PANCREATOBILIARY MALIGNANCY 50
MECHANISM OF CARCINOGENESIS 51
ENVIRONMENTAL POLLUTION, HEAVY METALS AND PANCREATO-BILIARY MALIGNANCY 52

2. AIM OF THE STUDY 61

3. MATERIALS & METHODS 65
SUBJECT AND STUDY DESIGN 65
BILE SAMPLE ANALYSIS 67
Collection, storage and handling of bile samples 67

4. PILOT STUDY 70
STANDARDISATION OF METHODOLOGY – THE INITIAL SAMPLES 70
Syringe filtration method 70
Acid digestion method 71
SPECTROMETRIC ANALYSIS 71
RESULTS OF PILOT STUDY – FIRST 25 SAMPLES 72
Acid digestion versus syringe filtration 73
Comparison between benign and malignant 76

5. MAIN STUDY 80
CALCULATION OF SAMPLE SIZE 80
RESULTS OF MAIN STUDY 83
AGE AND SEX DISTRIBUTION 86
AGE AND SEX DISTRIBUTION ACCORDING TO DIAGNOSIS 88
REGIONAL VARIATION 90
VARIATION IN LEVELS OF OBSTRUCTION TO THE BILIARY TREE IN DIFFERENT GROUPS 92

SOURCE OF BILE – COMMON BILE DUCT (CBD) OR GALLBLADDER (GB) – EFFECT ON BILIARY METAL CONCENTRATION 93
Zinc *94*
Copper *95*
Cadmium *96*
Lead *97*
OBSTRUCTED BILIARY SYSTEM – EFFECT ON BILIARY METAL CONCENTRATION 99
Zinc *100*
Copper *101*
Cadmium *102*
Lead *103*
COMPARISON BETWEEN GROUPS FOR EACH METAL – THE FINAL ANALYSIS 104
Zinc *107*
Copper *108*
Cadmium *109*
Lead *110*
6. DISCUSSION 113
SECRETORY AND EXCRETORY FUNCTIONS OF BILE 113
PHYSIOLOGY OF BILE SECRETION 114
HEAVY METAL EXPOSURE AND ASSOCIATION WITH CANCER 119
GENERAL MECHANISM OF METAL CARCINOGENICITY 125
Induction of oxidative stress *126*
Interference with DNA repair *127*
Deregulation of cell proliferation *128*
CADMIUM AND ITS ROLE IN PANCREATOBILIARY MALIGNANCY 128
ENVIRONMENTAL POLLUTION IN THE NORTH-EAST OF ENGLAND 135
7. CONCLUSION AND THE FUTURE OF THE PROJECT 147
8. SUMMARY OF WORK IN PUBLIC DOMAIN 151
9. APPENDIX I (CONSENT FORM) 152
10. APPENDIX II (INFORMATION SHEET FOR PATIENTS) 153
11. REFERENCE 154

LIST OF FIGURES

Figure 1.1: Morphological classification of biliary cancer (CCA – cholangiocarcinoma) 14

Figure 1.2: Bismuth-Corlette classification of biliary tumours 15

Figure 1.3: Regional differences in the mean estimated annual percentage change (EAPC) in age-adjusted (1970 World standard population) gender specific mortality rates from intrahepatic biliary tract tumours (top) and extrahepatic biliary tract tumours (bottom) (Patel, 2002) 22

Figure 1.4: Incidence of intra-hepatic and extra-hepatic cholangiocarcinoma in the United States from 1975 to 1999. Note that the term intrahepatic cholangiocarcinoma includes peri-hilar lesions (Lazaridis and Gores, 2005, Shaib and El-Serag, 2004)). 23

Figure 1.5: International age-standardized mortality rates (ASMR) for intra-hepatic cholangiocarcinoma (A) and for gall bladder and extra-hepatic cholangiocarcinoma (B) (Khan et al., 2005). 24

Figure 1.6: Proposed model for carcinogenesis in cholangiocarcinoma showing interaction between environmental factors and host genetics (Khan et al., 2005). 52

Figure 1.7: Cadmium, chromium and lead concentration in patients with gallstones and gallbladder cancer (Shukla et al., 1998b). 57

Figure 4.1: ATI Unicom SolaaR 929 atomic absorption spectrometer 72

Figure 4.2: Comparison of acid digestion (AD) and syringe filtration (SF) method for concentration of zinc (Zn) 73

Figure 4.3: Comparison of acid digestion (AD) and syringe filtration (SF) method for concentration of cadmium (Cd) 74

Figure 4.4: Comparison of acid digestion (AD) and syringe filtration (SF) method for concentration of copper (Cu) 75

Figure 4.5: Comparison of median concentration of lead (Pb) in the bile samples from patients with benign and malignant diseases 76

Figure 4.6: Comparison of median concentration of cadmium (Cd) in the bile samples from patients with benign and malignant diseases 77

Figure 5.1: Data entry form for present study (Microsoft Access©) 83

Figure 5.2: Histogram of the age distribution of the study population 86

Figure 5.3: Sex distribution of all the patients 87

Figure 5.4: Histological diagnosis (CA = Carcinoma) 88

Figure 5.5: Age distribution of individual groups of patients 89

Figure 5.6: Sex distribution according to diagnosis 90

Figure 5.7: Distribution of patients into North and South of the region according to diagnosis 91

Figure 5.8: Distribution of patient depending on presence of biliary obstruction (serum bilirubin level >35 µmol/L) 92

Figure 5.9: Graphical representation of correlation between zinc level of samples from gallbladder (GB) and common bile duct (CBD) 94

Figure 5.10: Graphical representation of correlation between copper level of samples from gallbladder (GB) and common bile duct (CBD): 95

Figure 5.11: Graphical representation of correlation between cadmium level of samples from gallbladder (GB) and common bile duct (CBD) 96

Figure 5.12: Graphical representation of correlation between lead level of samples from gallbladder (GB) and common bile duct (CBD)..... 97

Figure 5.13: Distribution of patient depending on presence of biliary obstruction (serum bilirubin level >35 µmol/L) 99

Figure 5.14: Graphical representation of correlation between bilirubin level and zinc concentration..... 101

Figure 5.15: Graphical representation of correlation between bilirubin level and copper concentration..... 102

Figure 5.16: Graphical representation of correlation between bilirubin level and cadmium concentration..... 103

Figure 5.17: Graphical representation of correlation between bilirubin level and lead concentration..... 104

Figure 5.18: Means plot of zinc, copper, cadmium and lead concentration in each group of patients..... 107

Figure 5.19: Box plot comparing median concentration of zinc between different groups of patients..... 108

Figure 5.20: Box plot comparing median concentration of copper between different groups of patients..... 109

Figure 5.21: Box plot comparing median concentration of cadmium between different groups of patients..... 110

Figure 5.22: Box plot comparing median concentration of lead between different groups of patients..... 111

Figure 6.1: Mechanisms of bile formation. Bile formation begins at the canalicular level ('canalicular bile'; 75% of daily bile production), followed by modifications along the bile ducts ('ductal bile'; 25%)(Ferenci et al., 2002)..... 114

Figure 6.2: Copper transport in human liver. Copper taken up from blood (via CTR) is taken up by metallothionein, which is the storage protein for a variety of metal ions including zinc and copper (Ferenci et al., 2002)..... 117

Figure 6.3: Global production and consumption of selected toxic metals, 1850–1990 120

Figure 6.4: Spatial distribution of natural cadmium emissions in Europe in 2007, g/km2/y 121

Figure 6.5: Spatial distribution of natural lead emissions in Europe in 2007, kg/km2/y 121

Figure 6.6: Cellular uptake, intracellular distribution and binding of soluble and particulate metal compounds (Beyersmann and Hartwig, 2008)..... 125

Figure 6.7: Metal ions and oxidative stress. Modified from (Beyersmann and Hartwig, 2008) 127

Figure 6.8: Estates selected for the analysis of mortality and survey data. Zone A, B and C indicate varying proximity to industry (Byrnes and Afdhal, 2002). 137

Figure 6.9: Metal mining areas of England and Wales 139

Figure 6.10: Map showing catchments of England and Wales with likely ('at risk and 'probably at risk') to have sediment-borne, mining related metal contamination (after Environment Agency, 2008)..... 143

Figure 6.11: Geomorphology and sediment-borne metal concentrations along River Swale, North-East England (Brewer et al., 2005) 144

Figure 6.12: Example of floodplain map showing probability of contamination along River Swale (Brewer et al., 2005)..... 144

LIST OF TABLES

Table 1.1: Classification of malignant tumours of gallbladder (Albores-Saavedra et al., 1986) 18

Table 1.2: Classification of tumours of exocrine pancreas and peripancreatic area(Kloppel and Luttges, 2001). 19

Table 1.3: Risk factors for cholangiocarcinoma 33

Table 1.4: Risk factors for pancreatic cancer and their interpretation by the cadmium hypothesis (Schwartz and Reis, 2000). 56

Table 5.1: Details of analyzed samples 85

Table 5.2: Age distribution of the study population 86

Table 5.3: Age distribution of individual groups of patients 88

Table 5.4: Sex distribution vs. diagnosis (Chi-Square Test) 90

Table 5.5: Distribution of patients from the North and South of the region depending on the post code 91

Table 5.6: Zinc level from samples taken from gallbladder (GB) and common bile duct (CBD) – correlation statistics 94

Table 5.7: Copper level from samples taken from gallbladder (GB) and common bile duct (CBD) – correlation statistics 95

Table 5.8: Cadmium level from samples taken from gallbladder (GB) and common bile duct (CBD) – correlation statistics 96

Table 5.9: Lead level from samples taken from gallbladder (GB) and common bile duct (CBD) – correlation statistics 97

Table 5.10: Correlation between bilirubin level and zinc concentration 100

Table 5.11: Correlation between bilirubin level and copper concentration 101

Table 5.12: Correlation between bilirubin level and cadmium concentration 102

Table 5.13: Correlation between bilirubin level and lead concentration 103

Table 5.14: Raw data of each metal concentration among different groups of patients 105

Table 5.15: Results of ANOVA to look at the differences between groups of patients 106

Table 6.1: Classification of metals and/or their compounds as carcinogenic. Source: International Agency for Research on Cancer ((IARC)) Monographs, MAK (German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area). (ø, not classified) 124

Table 6.2: River basins most affected by metal mining in England and Wales 140

Table 6.3: Dissolved metal concentration in water samples from some mining impacted English and Welsh rivers. Values in bold exceed the environment quality standard (EQS) (Hudson-Edwards et al., 2008) 142

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Abstract

Abstract

Introduction

Biliary and pancreatic cancers are associated with poor prognosis and high mortality. A variety of genetic and environmental factors have been implicated as possible causes of these cancers. New epidemiological studies are looking into the possible role of heavy metals such as lead, chromium, nickel and cadmium as carcinogens. Studies from areas of wide geographical differences such as Egypt, USA and India have reported a possible association of heavy metals such as lead and cadmium with pancreato-biliary malignancy. These studies were from areas of significant environmental pollution with these metals due to the proximity of heavy industry or mining activities. No association has been demonstrated between metal exposure and pancreato-biliary malignancy in areas of low environmental pollution.

Aim

The aim of this study was to look at a possible association between excretion of heavy metals from the biliary tract and pancreato-biliary malignancy in an industrial area in the North-East of England.

Materials and Methods

Patients with malignant biliary obstruction admitted to a tertiary referral centre were identified prospectively. Bile was collected either during endoscopic retrograde cholangiography (ERC), percutaneous transhepatic

cholangiography (PTC) or during biliary tract surgery. Bile was also collected from patients with benign biliary disease to act as controls. The bile samples were analyzed for concentrations of lead, copper, zinc, cadmium, chromium and nickel using atomic absorption spectrometry after pre-treatment by the acid digestion method. The mean levels of every metal in the study groups (biliary malignancy and pancreatic malignancy) and the control group were compared by using SPSS 16© (SPSS Inc) statistical software.

It was uncertain whether there would be a measurable quantity of heavy metals in the bile collected during the above procedures and therefore it was felt necessary to perform a small pilot study to standardize the methods and address any possible difficulties.

Results

Bile samples collected from 107 patients over a period of 18 months were included in this study. As part of the pilot study two different methods of purification of bile (acid digestion *vs* syringe filtration) were evaluated and acid digestion was chosen as a more reliable method. For the main study, after exclusion due to inadequate volume of sample, a total of 127 bile samples from 98 patients (43 male, 55 female, median age 65.82 years, range 20-82 years) were analyzed. On final analysis, the malignant group comprised of 3 subgroups: pancreatic cancer, biliary cancer and periampullary cancer. Results from these 3 groups were compared with those from the benign control group. The groups were sex-matched but there was a significant difference in age distribution between the benign and

malignant groups ($p<0.001$). Bile samples were obtained from both gallbladder (GB) and common bile duct (CBD) in 33 patients and from either of these sources in the remainder. There was a strong positive correlation between the concentration of each metal when obtained from either GB or CBD.

The main results of this study have shown that copper was present at significantly higher levels in the benign control group ($p=0.004$) and that cadmium was present at significantly higher levels in the malignant groups ($p=0.02$). There was no significant difference amongst the groups for the other heavy metals.

Discussion

Cadmium has been implicated as a possible environmental carcinogen for various malignancies, including pancreatic cancer. Most studies that show this association are from areas of high environmental pollution. The present study supports this association. There is evidence of excess environmental exposure to heavy metals, including cadmium, in the North-East of England. This exposure is mainly in relation to mining activities in the past in this region and is mainly noticed downstream along the rivers draining the areas with significant mining history. The results of this study support the possible association between heavy metal exposure and pancreatic and biliary malignancy. Further research is needed to establish the specific carcinogenic role of heavy metals such as cadmium in pancreato-biliary malignancies.

Chapter 1

Introduction

1. INTRODUCTION

Morphology of biliary and pancreatic cancer

Biliary and pancreatic cancers form a group of diseases that has always been challenging for the clinician. They present late and are difficult to diagnose. Once diagnosed, they carry a poor prognosis because surgery is the only curative option for most of these tumours and success of radical surgery depends on detecting them early. Effective adjuvant treatment for pancreato-biliary cancers is the subject of scientific research and clinical trials. Often described together because of their usual adverse prognosis, biliary and pancreatic cancers actually form a very heterogeneous group of malignancies with different aetiologies and clinical outcome.

For ease of discussion, pancreato-biliary cancers can be divided into the following broad headings:

1. Bile duct cancer
    a. Intra-hepatic (involving biliary tree within the liver)
    b. Extra-hepatic (involving biliary tree outside the liver)
        i. Hilar
        ii. Middle
        iii. Distal
2. Gallbladder cancer
3. Pancreatic cancer
    a. Pancreatic ductal carcinoma
    b. Peri-ampullary carcinoma

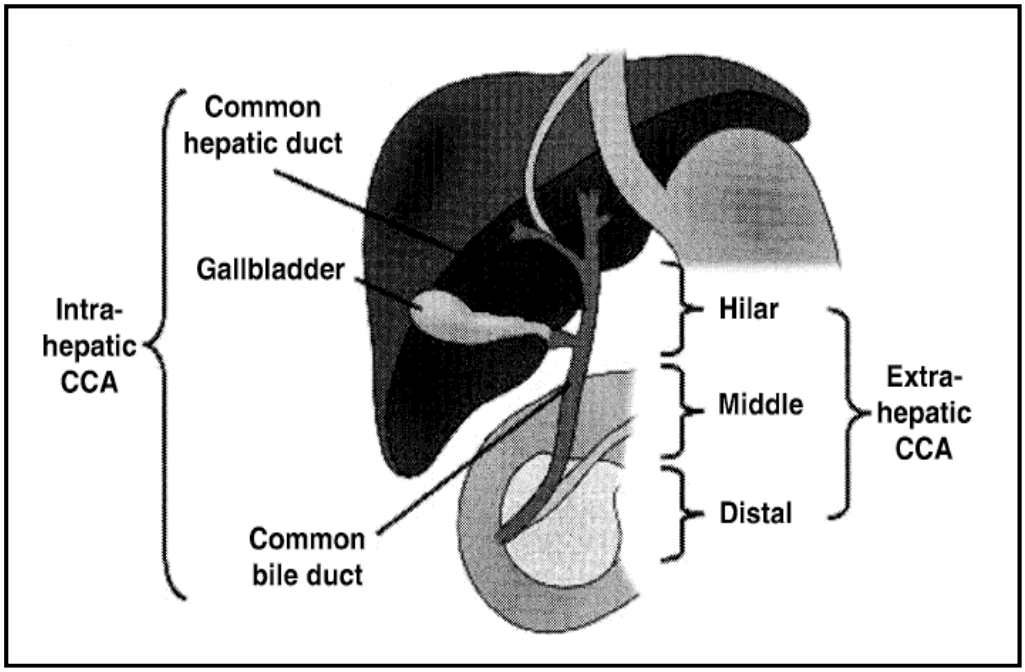

Figure 1.1: Morphological classification of biliary cancer (CCA – cholangiocarcinoma) (Lazaridis and Gores, 2005)

The above classification is entirely based on the anatomical location of the lesion in the biliary tree (Figure 1.1). Two thirds of all cholangiocarcinomas are of the extra-hepatic type and 60% of all extra-hepatic cholangiocarcinomas are of the proximal hilar type (Klatskin tumour) (Klatskin, 1965). This is again morphologically classified into several types by the Bismuth-Corlette classification (Figure 1.2).

- Type I: Tumours below the confluence of the left and right hepatic ducts.
- Type II: Tumours reaching the confluence but not involving left or right hepatic ducts.
- Type III: Tumours occluding the common hepatic duct and either the right (IIIa) or the left (IIIb) hepatic duct.
- Type IV: Tumours that are multi-centric or involving both right and left hepatic ducts.

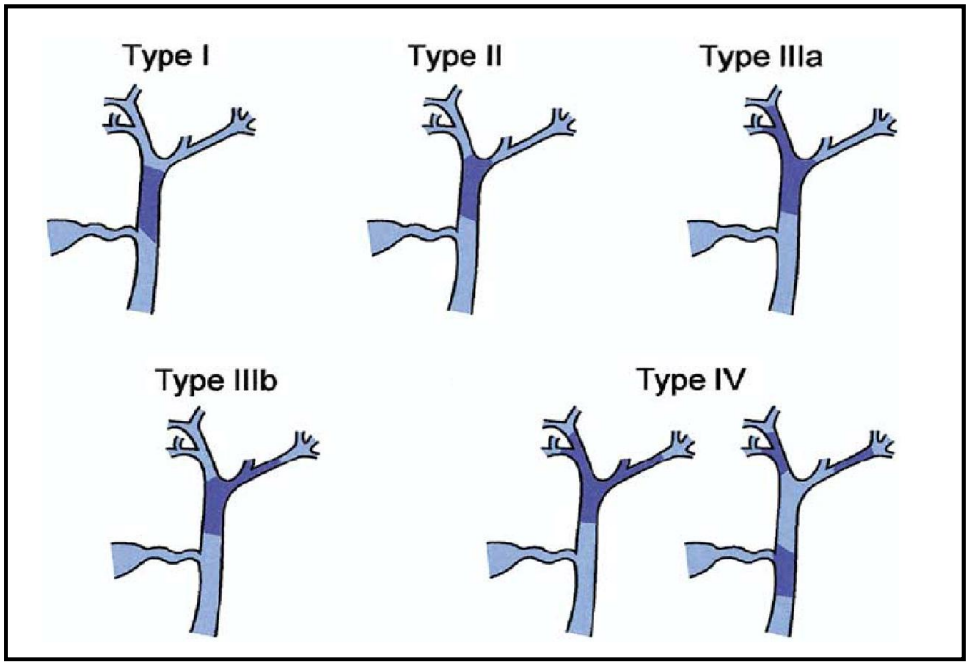

Figure 1.2: Bismuth-Corlette classification of biliary tumours

Pancreatic tumours can be classified into primary ductal tumours and peri-ampullary carcinoma depending on their origin. Although they are often grouped together and clinically present in a similar pattern, pancreatic ductal adenocarcinomas and peri-ampullary tumours have very different prognosis. Peri-ampullary tumours are classically defined as tumours arising from within 2 centimetres from the Ampulla of Vater. They can arise from epithelium of distal bile duct, duodenal mucosa or ampullary epithelium.

Pathology of biliary and pancreatic cancer

Cholangiocarcinoma

Tumours of the extra-hepatic biliary tree are found mainly in 3 sites.

1. Proximal to junction of cystic duct including the hilar region
2. Distal to cystic duct (mid third)
3. Lower end of common bile duct (distal third)

The usual appearance of proximal and hilar lesions (Klatskin 1965) (Klatskin, 1965) is that of a sclerosing with an increase in ductal wall thickness. It often extends into the surrounding liver tissue as a mass-forming lesion. Based on computerised tomographic (CT) scanning, several growth patterns have been proposed, i.e. infiltrative and exophytic and the latter is subdivided into nodular and periductal types. Occasionally, proximal tumours are polypoid and protrude into the lumen of the bile duct. Usually they can be clearly demarcated from the surrounding hepatic parenchyma on microscopy. Tumours in the mid third are generally discrete tumours, causing thickening of the ductal wall, but often presents as a sharply demarcated, spherical mass. The tumours in the distal 2cm of the common bile duct are papillary adenocarcinoma and are usually quite friable. Most ductal tumours fall into these three categories, with annular sclerosing tumours proximally, globular or nodular sclerosing type in the mid-region and papillary tumour at the lower third.

Microscopically the tumours are generally adenocarcinomas. Several histological variants of cholangiocarcinomas have been reported. They comprise distinct forms of adenocarcinomas (papillary, intestinal, mucinous, clear cell, signet cell and adenosquamous types), and squamous cell, small cell and undifferentiated carcinomas. A distinct variant associated with hepatolithiasis and recurrent pyogenic cholangitis is mucin-producing cholangiocarcinoma, which has a better prognosis.

Gallbladder Cancer

As with most tumours, gall bladder epithelium progresses from dysplasia to carcinoma in-situ to invasive carcinoma. Severe dysplasia and carcinoma in situ is identified in more than 90% of cases of gallbladder carcinoma. It is likely that areas of dysplasia and carcinoma in situ are missed in routine histological examination following cholecystectomy, as there are no associated macroscopic characteristics that would target an area for histological examination. The rate of progression of precursor lesions to invasive carcinoma is about 15 years (Albores-Saavedra et al., 1986).

Carcinomas are difficult to distinguish from chronic cholecystitis at early stages and are often found incidentally following routine cholecystectomy. Nearly 60% of tumours originate in the fundus of the gallbladder, 30% in the body and 10% in the neck (Albores-Saavedra et al., 1986). Macroscopically the tumours can be classified as infiltrative, nodular, combined nodular-infiltrative, papillary and combined papillary-infiltrative forms. The most common forms are the infiltrative or nodular-infiltrative forms.

The histological classification of malignant tumours of gallbladder is given in the following table. In general, malignant tumours of the gallbladder carry a very poor prognosis. Majority of them are adenocarcinoma and the other varieties are extremely rare (Table 1.1). The only histological type with definite prognostic significance is the papillary adenocarcinoma, which has significantly better survival compared to all other histological types.

Malignant epithelial tumours	Malignant mesenchymal tumour
Adenocarcinoma Well differentiated Papillary Intestinal type Pleomorphic giant cell Poorly-differentiated, small cell Signet ring cell Clear cell Squamous Adeno-squamous Oat cell carcinoma	Embryonal rhabdomyosarcoma Leiomyosarcoma Malignant fibrous histiocytoma Angiosarcoma

Table 1.1: Classification of malignant tumours of gallbladder (Albores-Saavedra et al., 1986)

Pancreatic and periampullary carcinoma

Pancreatic cancer, especially those involving the head of pancreas and periampullary region, is actually a combination of different pathological entities with different presentation, behaviour and outcome. The most common malignant pancreatic tumour is pancreatic ductal adenocarcinoma. However, it is often difficult to diagnose whether or not a lesion situated in the head of pancreas is an ampullary lesion, duodenal lesion, a distal bile duct lesion or an islet cell tumour. Cystic neoplasms, both serous and mucinous cystadenoma and cystadenocarcinoma are more common than previously suspected and should be proactively looked for so that they can be resected where appropriate. Pathological classification of pancreatic and periampullary tumours is given in the table (Table 1.2).

Benign pancreatic tumours	Cystadenoma Serous Mucinous Intraductal papillary-mucinous Cystic teratoma Adenoma Ampulla Duodenum
Malignant pancreatic tumours	Adenocarcinoma Mucinous Signet ring cell Adenosquamous Anaplastic Mixed ductal-endocrine Duodenal adenocarcinomas Ampullary carcinoma Distal bile duct carcinoma Cystadenocarcinoma Serous Mucinous Intraductal papillary Giant cell Acinar cell Pancreatoblastoma

Table 1.2: Classification of tumours of exocrine pancreas and peripancreatic area(Kloppel and Luttges, 2001).

Pancreato-biliary malignancy – prognosis & worldwide trends in mortality

Pancreato-biliary malignancies have always been a difficult disease to treat due to their late presentation, poor sensitivity to conventional adjuvant treatments after surgery and our inability to prevent and detect early tumour formation. Their aetiology also remains poorly understood. Cancer of the pancreas is an important cause of cancer related mortality in the western world and account for nearly 30,000 deaths in the United States per year. It probably has the worst survival amongst the major common malignancies; the average overall survival after diagnosis is less than 6 months and less than 2% patients survive up to 5 years (Bray et al., 2002).

Bile duct cancer or cholangiocarcinoma as a pathological entity was reported for the first time by Durand-Fardel in 1840 (Olnes and Erlich, 2004). Although it comprises only 10% - 15% of hepatobiliary neoplasms, its incidence is definitely increasing, especially in the Western world (Shaib and El-Serag, 2004, Gores, 2003, Khan et al., 2002). The disease is difficult to diagnose and the prognosis is poor due to late presentation and lack of effective non-surgical treatment (Khan et al., 2005). Most of the patients with cholangiocarcinoma present with unresectable disease and die within a year of diagnosis. Death is due to liver failure, recurrent sepsis secondary to biliary obstruction and cancer cachexia (Carriaga and Henson, 1995). Overall survival rate, including those undergoing curative resection, is poor, with less than 5% overall survival at 5 years, similar to pancreatic cancer. In spite of advances in diagnostic modalities and surgical treatment, the overall prognosis of this disease has not significantly changed in the last 30 years (Shaib and El-Serag, 2004). Cholangiocarcinoma arises from the ductular epithelium of either intrahepatic or extrahepatic biliary tree, including the gallbladder. Aetiology of this disease remains poorly understood. Risk factors seem to differ according to the anatomical location of the tumour. Intrahepatic cholangiocarcinomas are highly prevalent in certain regions like Thailand and other Southeast Asian countries (Green A, 1991). Because parasitic biliary infection is endemic to these regions, liver fluke infection and chronic biliary tract inflammation are considered strong risk factors for cholangiocarcinoma (Patel, 2002, Flavell, 1981, Haswell-Elkins MR, 1994).

Other possible aetiology, including genetic and environmental factors have been investigated but the actual pathogenesis remains obscure.

Although cholangiocarcinoma is a rare neoplasm, its incidence has increased in the past three decades (Shaib and El-Serag, 2004). A recent increase in intrahepatic, but not extrahepatic biliary tumours have been reported from the United States (Patel, 2001). Similar increases in incidence of the intrahepatic tumour have been reported from other parts of the world including Europe and China (Hsing AW, 1998, Taylor-Robinson et al., 2001). However, the majority of the new cases diagnosed continue to be the extrahepatic variety. In the United States, approximately 5000 new cases are diagnosed every year, two thirds of which are extrahepatic lesions (Lazaridis and Gores, 2005, Shaib and El-Serag, 2004). Recent studies have indicated that intrahepatic and extrahepatic cholangiocarcinomas have a distinct epidemiology and the epidemiology of gallbladder cancer also has some unique features differing from other extrahepatic cholangiocarcinomas. In the past this distinction was not very clearly understood and therefore the reported epidemiological observations may not be correct. The intrahepatic cholangiocarcinomas were often combined with primary hepatocellular carcinoma and therefore the global increase in incidence and mortality from intrahepatic cholangiocarcinoma may partly be due to recognition of this disease as a separate entity (Patel, 2002).

A review by Patel et al (Patel, 2002) showed that there has been a global increase in mortality from intrahepatic cholangiocarcinoma with the greatest

increase noted in the Americas, Oceania and Western Europe (Figure 1.3). Central and Northern Europe have seen a less marked increase in mortality and same was seen in Asia and Middle East. However, data from regions of high incidence of intrahepatic cholangiocarcinoma such as Thailand and other South-East Asian countries were not included in this analysis. It could be concluded from the study that there has been increase in mortality from this disease in areas of low prevalence.

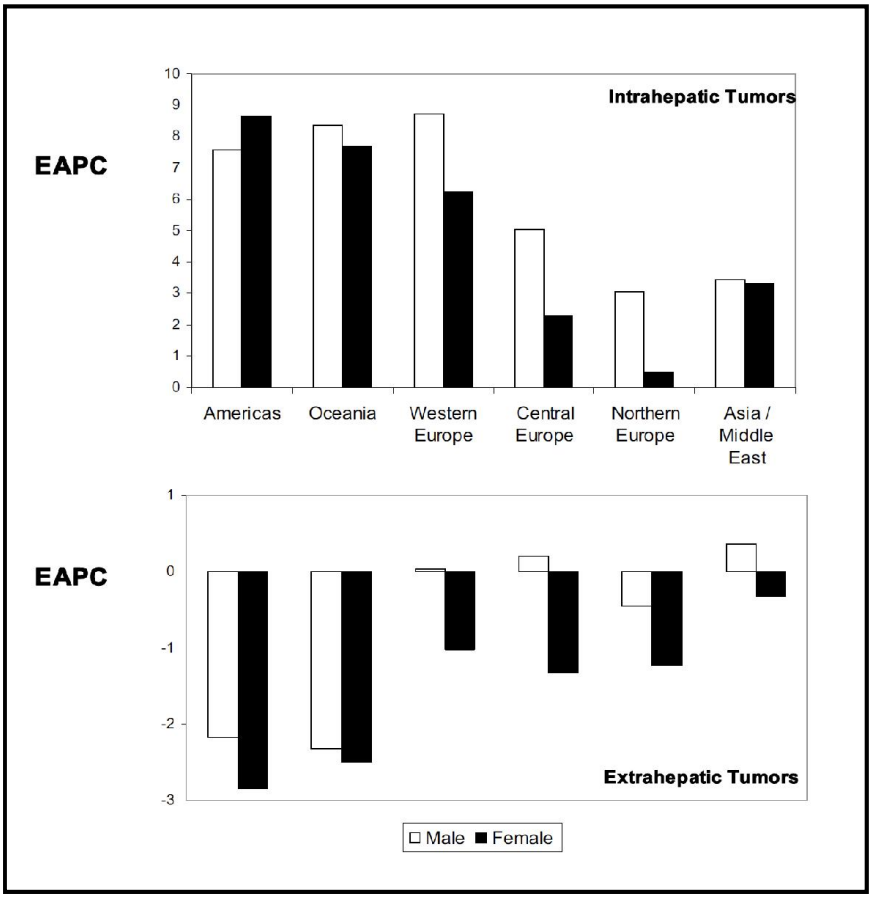

Figure 1.3: Regional differences in the mean estimated annual percentage change (EAPC) in age-adjusted (1970 World standard population) gender specific mortality rates from intrahepatic biliary tract tumours (top) and extrahepatic biliary tract tumours (bottom) (Patel, 2002)

Epidemiology

Cholangiocarcinoma

Cholangiocarcinoma accounts for 3% of all gastrointestinal cancers (Vauthey and Blumgart, 1994) and is the second commonest primary liver tumour (Khan et al., 2002, Vauthey and Blumgart, 1994). In the United States, the age-adjusted incidence of intrahepatic cholangiocarcinoma, the definition of which included peri-hilar lesions, increased from 0.32 in 100,000 in 1975-1979 to 0.85 in 100,000 in 1995-1999. On the other hand, the incidence of extrahepatic cholangiocarcinoma declined from 1.08 in 100,000 in 1979 to 0.82 in 100,1000 in 1982 (Shaib and El-Serag, 2004)(Figure 1.4). Several other studies have confirmed similar trends worldwide (Khan et al., 2002, Patel, 2001, Patel, 2002, Taylor-Robinson et al., 2001).

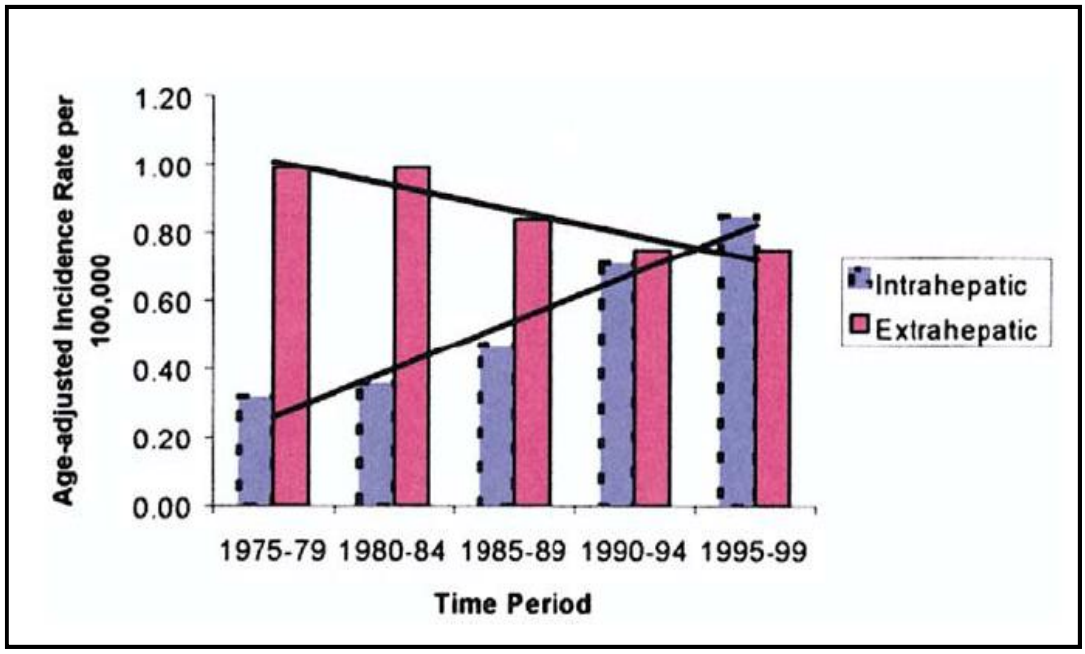

Figure 1.4: Incidence of intra-hepatic and extra-hepatic cholangiocarcinoma in the United States from 1975 to 1999. Note that the term intrahepatic cholangiocarcinoma includes peri-hilar lesions (Lazaridis and Gores, 2005, Shaib and El-Serag, 2004)).

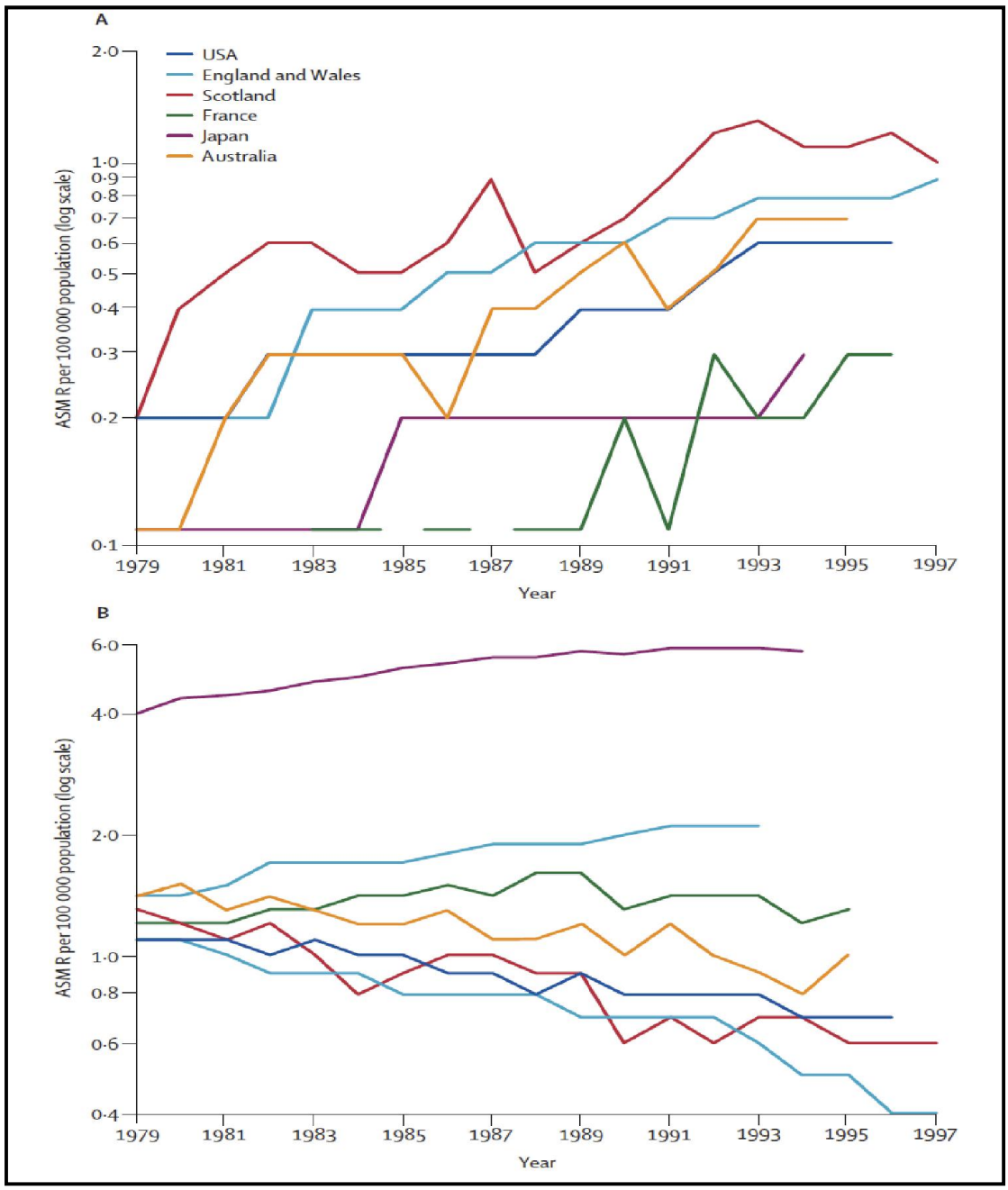

Figure 1.5: International age-standardized mortality rates (ASMR) for intra-hepatic cholangiocarcinoma (A) and for gall bladder and extra-hepatic cholangiocarcinoma (B) (Khan et al., 2005).

Intra-hepatic cholangiocarcinoma

The incidence of intra-hepatic cholangiocarcinoma shows significant variation worldwide. This probably reflects the influence of local environmental risk factors as well as genetic influences of different populations. The highest incidence is reported from north-east Thailand (96 per 100,000 men), other

South-East Asian countries and China, while Australia has a very low incidence of 0.2 per 100,000 men (Shaib and El-Serag, 2004). In recent years, an increase in incidence as well as mortality rate has been reported from all over the world including Europe, America and Australia (Khan et al., 2005) (Figure 1.5). In the United Kingdom, intra-hepatic cholangiocarcinoma has overtaken hepatocellular carcinoma as the leading cause of death from primary liver tumours (Khan et al., 2002, Taylor-Robinson et al., 2001). It needs to be investigated whether this increase in incidence is a true increase rather than one due to better detection or reclassification of hepatobiliary malignancy. Epidemiological studies from the United States show that age-adjusted incidence rates of intra-hepatic cholangiocarcinoma had increased by 165%, from 0·32 per 100 000 during 1975–79 to 0·85 per 100 000 during 1995–99 (Shaib et al., 2004). As with similar studies from other countries, this rise occurred across all age groups, especially in those older than 65 years, and in both sexes. Increased detection of a tumour is usually associated with an increase in the proportion of patients with early stage disease, or smaller sized lesions. The rise in intra-hepatic cancer was not associated with a significant change in the proportion of early stage cancers, histologically confirmed tumours, or smaller sized lesions(Shaib et al., 2004). Furthermore, the rising incidence does not seem to reach a plateau, as would be expected if it were due to an improvement in diagnostic modalities, such as endoscopic retrograde cholangiopancreatography (ERCP), MRI, or CT, which have become established practice for several years (Khan et al., 2002, Shaib and El-Serag, 2004, Taylor-Robinson et al., 2001). Also of note, the rise in intra-hepatic tumours is greater than the relative decline in extra-

hepatic tumours (Khan et al., 2002, Shaib and El-Serag, 2004). These factors suggest that the increasing incidence in intra-hepatic cancer is probably a true increase. However, the cause for increase in incidence of intra-hepatic cholangiocarcinoma remains unclear. The main risk factor for this disease in the western world is primary sclerosing cholangitis (PSC) and that has shown no significant increase in incidence. Primary sclerosing cholangitis is associated with the development of intra-hepatic cholangiocarcinoma in younger patients and the increase in incidence of intra-hepatic cholangiocarcinoma has mainly affected the older population (Khan et al., 2005, Shaib and El-Serag, 2004).

Extra-hepatic Cholangiocarcinoma & Gallbladder cancer

There are very few epidemiological data for extra-hepatic cholangiocarcinoma. However, incidence and mortality rates seem to be declining in many countries (Figure 1.5). Surveillance, Epidemiology and End Result (SEER) data from US National Institute of Health suggest that age-standardized mortality rates for extra-hepatic tumours fell from 0·6 per 100 000 in 1979 to 0·3 per 100 000 in 1998; and age-standardized incidence rates decreased from 1·08 per 100 000 to 0·82 per 100 000 in the same period (Patel, 2001, Shaib and El-Serag, 2004). Similar trends have been reported worldwide (Khan et al., 2002, Patel, 2002). The data for extra-hepatic cancer are perhaps more difficult to obtain because gallbladder cancers are often combined with extra-hepatic cholangiocarcinoma for ICD coding purposes, and gallbladder cancer incidence is known to be falling, probably as a result of increasing cholecystectomy rates over the past few decades (Khan et al., 2002, Taylor-Robinson et al., 2001).

Carcinoma of the gallbladder is a rare malignancy, usually associated with late presentation and therefore carries a poor prognosis. Non-metastatic lesions carry a 5-year survival rate of 32%, whereas more advanced stages have a 1-year survival rate of only 10% (Lazcano-Ponce et al., 2001). It affects 1-2 people per 100,000 population in the United States, accounting for 2000 to 5000 new cases diagnosed each year (de Groen et al., 1999, Fong and Malhotra, 2001). The incidence of this varies in different parts of the world, with the highest incidence and mortality occurring in northern India, northeastern Europe, and in the native population in North & South America. Ethnicity influences the incidence of this disease. In the United States, western Native Americans and Hispanic Americans are at an increased risk compared to the general US population, while African Americans are at an equivalent or slightly decreased risk compared to the Caucasian population (Lazcano-Ponce et al., 2001). Increased risk is also noted in women in Japan & Israel (Pandey and Shukla, 2003). The ratio between affected women to men also varies according to region, ranging between 1.5:1 and 6:1 (Lazcano-Ponce et al., 2001, Misra et al., 2003, Yee et al., 2002). The risk increases with age, with the maximum incidence occurring in the 7th decade (Fong and Malhotra, 2001).

Pancreatic Cancer

The incidence of pancreatic cancer has increased steadily in many countries for the last century. Mortality doubled in the UK between 1930 and 1970, but has risen much more slowly since then and it is the sixth most common cancer death in this country. The incidence is higher in Western or industrialized countries in general. Pancreatic cancer is rare before the age

of 45 years and 80% of cases occur in the 60–80 year age group. A study in the West Midlands indicated an age standardised incidence between 1960 and 1984 of approximately 10 cases per 100 000 population. There seems to have been some plateau of the annual incidence reported in this and other series. Because the five-year survival of this condition is so poor, incidence and mortality rates are virtually identical. Pancreatic cancer has been more common in men than women but this is trend is now beginning to change. In the USA, the Surveillance, Epidemiology, and End Results (SEER) program has shown a fall in the total incidence of pancreatic cancer from 12.3 per 100, 000 in 1973 to 10.7 per 100 000 in 1999. During the same period, the decline in rates for men was from 16.1 per 100 000 population to 12.1 per 100 000, and for women from 9.6 per 100 000 to 9.5 per 100 000, respectively.

Other periampullary tumours (of the ampulla, lower common bile duct, or duodenum) can present with similar symptoms and signs to pancreatic cancer. Without careful histological evaluation the differential diagnosis of tumour types may be impossible. The numbers of periampullary cancers are lower than pancreatic cancers, but they are more often resectable, so as many as half of pancreatic resections are for these periampullary tumours.

Risk factors for pancreato-biliary malignancy

Cholangiocarcinoma

Most of the identified risk factors for cholangiocarcinoma are associated with chronic inflammation and scarring of the biliary epithelium.

Primary sclerosing cholangitis

Primary sclerosing cholangitis is the commonest predisposing condition for cholangiocarcinoma in the West. Cholangiocarcinoma rates of 8–40% have been reported in patients with primary sclerosing cholangitis in follow up studies and liver explant specimens (Shaib and El-Serag, 2004). Cholangiocarcinoma in such patients tends to present earlier, in 30–50 year age-groups, than in sporadic cases (Pitt et al., 1995). About a third of patients with primary sclerosing cholangitis who develop cholangiocarcinoma do so within two years of diagnosis and the risk of developing cholangiocarcinoma seems unrelated to the duration of the inflammatory disease (Bergquist et al., 1998, Broome et al., 1996). Two-thirds of patients with primary sclerosing cholangitis have associated inflammatory bowel disease, especially ulcerative colitis (Broome et al., 1996). No association has been shown between the risk of cholangiocarcinoma and the presence, severity, and extent of inflammatory bowel disease in this group of patients (Broome et al., 1996, Chalasani et al., 2000).

Parasitic Infection

Experimental and epidemiological data suggest a pathogenic association between liver fluke infestation, especially *Opisthorcis viverrini* (and less definitively *Clonorchis sinensis*) and cholangiocarcinoma (Watanapa and Watanapa, 2002). Most epidemiological data are from Thailand, which has the highest incidence of cholangiocarcinoma worldwide (87 per 100 000 population) and where an estimated seven million people have opisthorchiasis (Watanapa, 1996). Eating undercooked fish infects human beings with adult worms inhabiting and laying eggs in the biliary system.

Apart from a strongly positive correlation between liver flukes and cholangiocarcinoma in case-control studies (Parkin et al., 1991), malignant change in the biliary epithelium of Syrian hamsters has been shown after infection with *O viverrini*, especially if fed nitrosamines. These carcinogens are produced by bacteria in fish and other foods and are thought to act as a cofactor in cholangiocarcinogenesis (Khan et al., 2005).

Fibropolycystic liver disease

Congenital abnormalities of the biliary tree associated with Caroli's disease, congenital hepatic fibrosis, and cystic dilatations of the bile ducts (choledochal cysts) carry a 15% risk of malignant change after the second decade, at an average age of 34 years. The overall incidence of cholangiocarcinoma in patients with untreated cysts is up to 28% (Lipsett et al., 1994, Scott et al., 1980). The mechanism of carcinogenesis is unclear, but could be related to biliary stasis, reflux of pancreatic juice causing chronic inflammation, activation of bile acids and deconjugation of carcinogens (Ohtsuka et al., 2001). Benign lesions like bile duct adenomas and biliary papillomatosis are also associated with the development of cholangiocarcinoma. Bile salt transporter protein polymorphisms in BSEP, FIC1, and MDR3 genes can lead to unstable bile content and to deconjugation of xenobiotics, previously conjugated in the liver (Jacquemin, 2001, van Mil et al., 2001). In the presence of congenital bile duct abnormalities, this process can lead to the development of cholangiocarcinoma at an early stage in life. It is possible that individuals who are heterozygous for bile salt transporter polymorphisms have an increased predisposition to cholangiocarcinoma as adults, after exposure to

cofactors that result in chronic inflammation in the biliary tree (Khan et al., 2005).

Intra-hepatic biliary stones

Intra-hepatic biliary stone is rare in the west, but relatively common in parts of Asia, and is associated with intra-hepatic cholangiocarcinoma which are usually peripheral in anatomical location (Shaib and El-Serag, 2004). Up to 10% of patients with hepatolithiasis develop cholangiocarcinoma (Kubo et al., 1995). In Taiwan, up to 70% of patients with cholangiocarcinoma undergoing resection have intra-hepatic biliary stones, and in Japan this figure is 6–18% (Okuda et al., 2002). The mechanism or carcinogenesis is thought to be bile stasis, predisposing to recurrent bacterial infections and subsequent inflammation, a potential cofactor for development of cholangiocarcinoma.

Chemical carcinogen exposure

Several chemical carcinogens have been associated with cholangiocarcinoma. Pro-mutagenic DNA adducts have been identified in cholangiocarcinoma tissue, indicating exposure to DNA-damaging agents (Khan et al., 2003). Thorotrast, a radiological contrast agent in use till 1960s and banned for its carcinogenic properties, has been strongly associated with the development of cholangiocarcinoma many years after exposure, increasing the risk to 300 times that of the general population (Sahani et al., 2003, Shaib and El-Serag, 2004). Associations have also been made with exposure to by-products from the rubber and chemical industries, including dioxins and nitrosamines (Hardell et al., 1984), as well as with alcohol and smoking (Bergquist et al., 1998, Chalasani et al., 2000), but results have been conflicting and no firm conclusions have been established.

Viral hepatitis

Cirrhosis has also been associated with cholangiocarcinoma (Shaib et al., 2005, Sorensen et al., 1998). A cohort study of over 11,000 patients with cirrhosis, followed up over 6 years, showed a 10-fold risk compared with the general population (Sorensen et al., 1998). More specifically, hepatitis B and C viruses have been linked to the cancer. A case-control study from Korea reported that 12·5% of patients with cholangiocarcinoma tested positive for hepatitis C virus and 13·8% for hepatitis B virus surface antigen (HBsAg), compared with 3·5% and 2·3% of controls (Shin et al., 1996). In a second case-control study from Italy, 23% of patients with cholangiocarcinoma were positive for antihepatitis C virus and 11·5% were HbsAg-positive compared with 6% and 5·5% of controls, respectively (Donato et al., 2001). A prospective controlled study from Japan reported the risk of developing cholangiocarcinoma in patients with cirrhosis related to hepatitis C virus as 3·5% at 10 years, 1000 times greater than in the general population (Kobayashi et al., 2000). Hepatitis C virus is an established risk factor for hepatocellular carcinoma and both hepatocytes and cholangiocytes have the same progenitor cell, supporting a role for the virus in carcinogenesis of both types of cancer. Furthermore, RNA from hepatitis C virus has been identified in cholangiocarcinoma tissue. A case-controlled study of risk factors for intra-hepatic cholangiocarcinoma from the United States showed adjusted odds ratios of 6·1 for hepatitis C virus, and 5·9 for HIV infection. Diabetes was also prevalent in intra-hepatic tumours (adjusted odds ratio 2·0), but pathogenic mechanisms are unclear (Shaib et al., 2005).

Biliary-enteric bypass procedures

In patients with biliary-enteric drainage procedures, reflux of intestinal contents into the biliary tree can occur and cause cholangitis. It will be reasonable to expect that recurrent cholangitis may lead to biliary carcinogenesis on long-term follow up. Tocchi et al reviewed more than 1000 patients undergoing three different procedures for biliary-enteric anastomosis (transduodenal sphincteroplasty, choledochoduodenostomy and hepaticojejunostomy) between 1967 and 1997 with a mean follow up of 129.6 months. The overall incidence of bile duct carcinoma was 5.5% with the maximum incidence being in the group undergoing choledochoduodenostomy (7.6%). Cholangitis was an independent factor affecting the incidence of cholangiocarcinoma (Tocchi et al., 2001). A list of known risk factors is given in Table 1.3 below.

- **Age >65 years**
- **Primary sclerosing cholangitis**
- **Liver fluke infestation (i.e. *Opisthorchis viverrini*)**
- **Choledochal cyst**
- **Caroli's disease**
- **Bile duct adenoma and biliary papillomatosis**
- **Hepatolithiasis**
- **Viral hepatitis and cirrhosis**
- **Chemical carcinogens (i.e. thorotrast)**
- **Surgical biliary-enteric anastomosis**

Table 1.3: Risk factors for cholangiocarcinoma

Gallbladder carcinoma

Gallbladder carcinoma has been linked to several risk factors although a direct causative role has not been established with most of them.

Cholelithiasis

The frequency of gallstones in the general population of the USA, as evidenced by the Framingham study, is 11% (Hart et al., 1971). The reported incidence of the association of gallstones in different series ranges from 40% to 100% (Adson, 1973, Chao and Greager, 1991, Hart et al., 1972, McLaughlin, 1964, Piehler and Crichlow, 1978, Shukla et al., 1985). Carcinoma of the gallbladder is extremely rare in the Bantu population, which is rarely affected by cholelithiasis (Pandey, 2003). There is a high incidence among Southwestern American Indians; symptomatic cholelithiasis has also been found to occur two to three times more frequently and at an earlier age in this population than in Caucasians (Reichenbach, 1967). Twenty-five per cent of carcinoma, however, occurs without documented cholelithiasis (Piehler and Crichlow, 1978). Kijima et al. (1989) suggested that chronic trauma and inflammation of the gallbladder mucosa, caused by the presence of gallstones, might induce epithelial dysplasia, which may predispose to carcinoma (Kijima et al., 1989, Piehler and Crichlow, 1978). However, if this hypothesis is true, one should see a higher incidence of squamous carcinoma rather then adenocarcinoma as malignant transformation in dysplasia gives rise to squamous carcinoma as seen at various other sites such as the cervix, the oral cavity and the oesophagus. The size of calculi was found to correlate with the probability of developing gallbladder carcinoma (Diehl, 1983). Comfort et al. (1948) followed silent gallstones for 10–25 years; less than 1% of patients developed carcinoma (Comfort et al., 1948). In another prospective randomized trial, 123 patients with gallstones were followed for more than 1000 person years; none of the patients

developed carcinoma (Gracie and Ransohoff, 1982). The clinical data do not establish a causative role for the gallstones, although the association is frequent enough to suggest a common antecedent or at least a facilitative role.

Racial and ethnic factors

There is a marked geographical difference in the distribution of carcinoma of the gallbladder. The observation of a high prevalence of carcinoma of the gallbladder in certain population groups led to speculation about the possible role of racial and ethnic factors in carcinogenesis. Sievers and Marquis (1962) found carcinoma of the gallbladder to be the second most common disease of the gastrointestinal tract in American Indians (Sievers and Marquis, 1962). Richenbach (1967) reported a six times higher incidence in Southwest American Indians than in the non-Indian population (Reichenbach, 1967). Rudolph et al. (1970) found carcinoma in 4.5–6.0% of American Indians undergoing cholecystectomies (Rudolph et al., 1970). Krain (1972) reported a high incidence of carcinoma of the gallbladder in Japanese people and Hart et al. (1972) found an incidence of 2.7/100 000 among the Israeli population; a high incidence was also found in European born women who later migrated to Israel (Hart et al., 1972, Krain, 1972). Klein and Finck (1972) found carcinoma of the gallbladder to be two times more frequent in the Mexican population of Southwest America (Klein and Finck, 1972). Shukla et al. (1985) reported an incidence of 0.3% of total hospital admissions in the Varanasi region of India (Shukla et al., 1985). Klein and Finck (1972) reported abnormal hepatic bile with a low ratio of bile acid and lecithin to cholesterol among Southwest American Indians both with

and without gallstones (Klein and Finck, 1972). Davion et al. (1989) reported racial differences in gallbladder motor functions among North American Indians and black Africans and suggested a possible role in cholelithiasis (Davion et al., 1989). Social and economic factors affecting dietary intake and environmental factors may also be responsible for this wide geographical variation observed in various groups with carcinoma of the gallbladder. However, this hypothesis needs to be tested in case–control and cohort studies.

Familial occurrence

The greater risk of developing the same cancer as a first degree relative, for breast, stomach, colon and prostate cancers is well established (Lynch, 1969). Patients suffering from gallbladder carcinoma are known to develop a second malignancy in 15% of cases and a family history of cancer of another organ is present in 33% of cases (Perpetuo et al., 1978). The first report of familial occurrence of carcinoma of the gallbladder came from two families of Hispanic New Mexicans (Devor and Buechley, 1980). The only other report is from Brazil (Trajber et al., 1982). Although three cases of familial linkage have been reported to date, the exact role of genetic factors in the causation of gallbladder carcinoma is still not clear.

Biliary tract anomalies

The anomalous junction of the pancreaticobiliary duct system is a congenital defect, characterized by the union of a common bile duct outside the duodenal wall. Because of the action of the sphincter muscle, this does not affect the union functionally but it is associated with various complications such as cholangitis, gallstones, biliary tract cancers, pancreatitis and

pancreaticolithiasis (Committee of Diagnostic Criteria of the Japanese Study Group on Pancreaticobiliary Maljunction, 1991). Attention has recently been focused on the high incidence of cancer of the biliary tract in patients with this anomaly (Aoki et al., 1987, Kimura et al., 1985, Kinoshita et al., 1984, Nagata et al., 1986). The incidence of malignant changes has been reported to be 15–40% (Aoki et al., 1987, Kimura et al., 1985, Ohta et al., 1990). There is an especially high incidence of cancer of the gallbladder in the anomalous junction without bile duct dilatation (Ohta et al., 1990). The incidence of gallstones in patients with carcinoma of the gallbladder associated with the anomalous junction is very low (Aoki et al., 1987). The reflux of pancreatic juice into the gallbladder with consequent chronic cholecystitis with intestinal metaplasia, a precancerous condition leading to differentiated carcinoma, has been implicated and the concentration of bile within the gallbladder of these patients may also promote gallbladder carcinogenesis (Nagata et al., 1985, Komi et al., 1982).

Chronic cholecystitis

The observation by Piehler and Crichlow (1978) that 40–50% of patients with gallbladder carcinoma had a history of antecedent chronic cholecystitis led to the theory that chronic cholecystitis per se is a causative factor in pathogenesis. However, the frequent association of stones precludes isolation of the effect of inflammation (Piehler and Crichlow, 1978). Calcification of the wall of the gallbladder, the so-called porcelain gallbladder, is believed to be an end stage of chronic cholecystitis. The incidence of carcinoma in calcified gallbladder is 12.5–61%, indicating that this is a high-risk group for developing carcinoma (Polk, 1966, Berk et al., 1973).

Xanthogranulomatous cholecystitis

This refers to lipids accompanying bile pigments and entering the connective tissue of the gallbladder wall, resulting in an inflammatory process. This lipid is sometimes oxidized to a coloured chromolipid and the condition is called ceroid granuloma (Amazon and Rywlin, 1980), ceroid-like histiocytic granuloma (Takahashi et al., 1976) or fibroxanthogranulomatous inflammation (Mehrotra and Bhatnagar, 1982). These terms now have been replaced by xanthogranulomatous cholecystitis (Mehrotra and Bhatnagar, 1982), a descriptive term first used by McCoy et al. (McCoy et al., 1976). The incidence of xanthogranulomatous cholecystitis ranges from 0.7% in Illinois, USA (Reyes et al., 1981) to 1.8% in Sheffield, UK (Benbow, 1990) with Japan having an intermediate rate of 1.2% among routine cholecystectomy specimens (Takahashi et al., 1976), although recent studies from Japan claim an incidence of 9% (Hanada et al., 1987). Not only does xanthogranulomatous cholecystitis mimic carcinoma in various ways, but there appears to be a possible association between the two. Gallbladder carcinoma was over-represented in a series of patients with xanthogranulomatous cholecystitis from Sheffield and the condition was more frequent than expected in series from Manchester, UK (Benbow, 1990, Houston et al., 1994). The reason for this association is not clear. However, it may simply be that xanthogranulomatous cholecystitis and adenocarcinoma are both complications of cholelithiasis and cholecystitis of a particular duration or degree, or it could be due to the tissue disruption by the carcinoma facilitating the entry of bile into the stroma (Benbow, 1989). The association, nevertheless, is important as both lesions are represented in the

same specimen and there is always a possibility of overlooking the carcinoma altogether (Benbow and Taylor, 1988).

Bacterial infections

The role of anaerobic and aerobic bacteria in biliary tract disease is well investigated (Flemma et al., 1967). Among aerobes, Escherichia coli, other coliform bacilli and enterococci are frequently encountered. Clostridium perfringens is the most common anaerobic organism along with anaerobic gram-positive cocci, B. fragilis, lactobacilli and actinomycetes in a small number of cases (Shimada et al., 1977). Dawson and Isselbacher (1960) demonstrated the toxicity of unconjugated bile acids and reported that unconjugated deoxycholate at a concentration of 5 $X10^{-3}$M causes dissolution of mucous membranes with loss of villi (Dawson and Isselbacher, 1960). Anaerobic bacteria deconjugate bile acids very effectively whereas aerobic bacteria, except Streptococcus feacalis and few other species, are inactive suggesting a possible role of bacterial deconjugation in tumor genesis (Shimada et al., 1969). Lowenfels (1978) proposed that in patients with gallstones the secondary bacterial infection releases cancer initiators or promoters from bile (Lowenfels, 1978). Kinoshita and Gelboin (1978) reported the formation of highly reactive intermediates during glucuronidase action of biliary glucuronidase, which bind to DNA and so are potentially carcinogenic (Kinoshita and Gelboin, 1978). Shukla et al. (1993) reported an increased bacterial degradation of primary to secondary bile acids, which are potent tumour initiators and promoters (Shukla et al., 1993). They suggested this to be a possible mechanism of carcinogenesis in patients with gallstones

Typhoid carriers

The production of tumour promoters and initiators by bacterial degradation of bile in the gastrointestinal tract is well established (Hill and Cook, 1986). An association of the chronic typhoid carrier state and carcinoma of the gallbladder was first reported by (Axelrod et al., 1971). (Welton et al., 1979)observed an excess of cancer of the hepatobiliary system in typhoid carriers; this was later confirmed by other studies (el-Zayadi et al., 1991, Mellemgaard and Gaarslev, 1988). Caygill et al. (1994) studied cancer mortality in people infected during the Aberdeen typhoid outbreak in 1964, in those who did not become carriers and in the carriers registered with the communicable disease unit in Scotland. They found increased mortality from cancers in the chronic typhoid carriers concluding that progression to chronic carrier states and not typhoid infection per se carries the excess cancer risk; and in the hepatobiliary system the main risk appears to be in the gallbladder. Their results suggested a lifetime risk of developing gallbladder cancer in 6% of the typhoid carriers (Caygill et al., 1994). An increased carrier rate among patients with gallbladder cancer has also been reported by Singh et al. and Shukla et al. from India (Singh et al., 1996).

Ulcerative colitis

The association between ulcerative colitis and biliary tract diseases is well known (Converse et al., 1971). Carcinoma of the extra-hepatic biliary tract is 5–10 times more frequent in patients with ulcerative colitis than in the general population (Ritchie et al., 1974, Converse et al., 1971). The first report of a biliary tract tumour and ulcerative colitis occurring in the same patient was made by Parker and Kendall (Parker and Kendall, 1954). The majority of

these carcinomas are of the bile duct; eight patients with carcinoma of the gallbladder have been reported (Warren et al., 1968, Babb et al., 1970, Converse et al., 1971, Morowitz et al., 1971, Ritchie et al., 1974, Herzog and Goldblum, 1996). The cause of these malignant lesions is not known. They frequently occur without pre-existing hepatobiliary disease and have been known to occur up to 53 years after total colectomy. Carcinoma of the gallbladder in association with ulcerative colitis therefore seems to have a different pathogenesis. A causative role of altered bile metabolism is only speculative (Dawson and Isselbacher, 1960, Piehler and Crichlow, 1978). Reproductive and hormonal factors The overall increased frequency of gallbladder carcinoma in women and bile duct cancer in men suggests a possible role for hormonal factors (Silk et al., 1989). The role of women's hormones in the formation of cholesterol gallstones is well established (Bennion and Grundy, 1978). A case–control study within the framework of a programme for studying risk factors in relation to cancer (Surveillance on Environmental Aspects in Relation to Cancer in Humans (SEARCH) programme) established by the International Agency for Research on Cancer ((IARC)) has shown that a younger age at menarche, an early age at first pregnancy, a higher number of pregnancies and prolonged fertility may enhance the risk of extrahepatic biliary tract malignancies (Plesko et al., 1985, Lambe et al., 1993, Moerman et al., 1994, Zatonski et al., 1997). The exact role of women's hormones is not yet clear, however, overall increased exposure to endogenous oestrogens and progesterone continues to be a high risk factor (Yen et al., 1987).

Dietary and life-style factors

The role of dietary factors in gallbladder cancer has been evaluated previously in several studies (Strom et al., 1995, Moerman et al., 1995, Moerman et al., 1997, Zatonski et al., 1992, Zatonski et al., 1997, Pandey and Shukla, 2002). An increased risk has been observed with obesity and a diet high in cholesterol, protein, carbohydrate and meat, while a diet high in fibre, vitamins C and E, fruits and vegetables appears to lower the risk. Life-style factors such as smoking and drinking alcohol have been found to be associated with obesity, gallstones and the risk of developing gallbladder and extra-hepatic bile duct cancers in earlier case–control studies (Moerman et al., 1997, Tseng et al., 1999). However, due to small numbers, it is difficult to derive any possible association. It appears to indirectly increase the risk, probably by increasing the incidence of gallstones and obesity. Benign neoplasms of the gallbladder have been identified in 0.15–8.5% of resected gallbladders (Piehler and Crichlow, 1978). The increased use of ultrasonography as the investigation of choice in patients with biliary symptoms has led to the increased detection of benign neoplasms, which until the last decade were considered to be of little significance and unlikely to undergo malignant change (Sawyer, 1970, Aldridge and Bismuth, 1990). Christenson and Ishak reviewed and classified 180 gallbladder tumours and found three cases of carcinoma in situ associated with non-papillary adenoma (Christensen and Ishak, 1970). Evidence for an adenoma–adenocarcinoma sequence comes from the study by Kozuka et al., who reviewed 1605 gallbladders and found seven adenoma with malignant changes and 79 invasive carcinomas; all the benign adenomas were less

than 12mm in diameter. All adenomas showing malignant changes were over 12mm and most invasive carcinomas were over 30 mm. There was a preponderance among women for both adenomas and invasive carcinomas (Kozuka et al., 1982). The need to define the precise relationship between the benign gallbladder neoplasm and the carcinoma was emphasized by Aldridge and Bismuth (Aldridge and Bismuth, 1990). Only five cases of carcinoma of the gallbladder developing in adenomyomatosis have been reported in the literature (Kawarada et al., 1986, Paraf et al., 1987, Katoh et al., 1988, Aldridge et al., 1991), despite the fact that adenomyomatosis constitutes 40% of benign gallbladder neoplasms. From the work undertaken by various authors there appears to be an increased risk of malignant transformation in some benign lesions like adenomas and it could be one of the mechanisms of carcinogenesis.

Carcinogens

The chemical similarity of the carcinogen methyl cholanthrene to naturally occurring bile acids has led to speculation that carcinoma of the gallbladder could be caused by such chemical transformation, in vivo. Methyl cholanthrene has not been identified as such in bile of patents with carcinoma (Gradisar and Kelly, 1970, Alonso de Ruiz et al., 1982, Klamer and Max, 1983, Chao and Greager, 1991). Fortner and others were able to induce cancer by implanting pellets of chemicals into gallbladders of cats and dogs (Fortner and Leffall, 1961, Fortner, 1955a, Fortner, 1955b). Simmers and Podolak failed to produce carcinoma by inserting methyl cholanthrene pellets in gallbladders of guinea pigs, establishing a significant species

difference (Simmers and Podolak, 1963). The introduction of foreign bodies into the gallbladder is also known to cause carcinogenesis. Petrov and Krotkina induced carcinoma by inserting glass rods into guinea pig gallbladders (Petrov and Krotkina, 1947). Feeding numerous other chemicals including O-aminoazotoluene and various nitrosamines has induced carcinoma of the gallbladder (Erturk et al., 1970, Enomoto et al., 1974, Kelly and Chamberlain, 1982, Klamer and Max, 1983). Cryer and Kissane linked biliary tract cancer to benzidine, 3,3-dichloro benzidine and M-toluenediamine. Kowalewski and Todd reviewed the probable multifactorial aetiology of this disease by inserting cholesterol pellets into the gallbladders of hamsters and later feeding them dimethyl nitrosamine; 67% developed cancer compare to 6% of control animals fed on carcinogen only (Kowalewski and Todd, 1971).

Secondary bile acids

Interest in the hypothesis that bile acids are possible precursors of carcinogenic aromatic hydrocarbons has now faded. However, there is a large body of evidence to suggest that bile acids are tumour promoters and co-mutagens (Reddy et al., 1976, Wilpart, 1991), can cause dysplastic changes in colonic mucosa (Wargovich et al., 1983, Castleden et al., 1989, Rafter and Branting, 1991) and are strongly implicated as tumour promoters in colon carcinogenesis (Hill, 1991, Hill, 1983) and in gastric cancer (Houghton et al., 1986). Bile acids are also reported to influence the growth and morphology of cultured human fibroblasts (Trias et al., 1977) and to promote hepatoma formation in rats. Shukla et al. reported higher levels of

secondary biliary bile acids in patients with carcinoma of the gallbladder, suggesting a possible role in gallbladder carcinogenesis (Shukla et al., 1993). Pandey et al. demonstrated increased degradation of primary to secondary bile acids by bacterial degradation (Pandey et al., 1995).

Free radicals

Free radical mechanisms are increasingly being implicated in almost all disease states (Del Maestro et al., 1980, Bulkley, 1983, Halliwell and Gutteridge, 1984, Halliwell, 1989, Reilly and Bulkley, 1990, Sinclair et al., 1990). Their relationship to neoplastic transformation in particular has attracted much attention (Breimer, 1988). Biological oxidation involves the generation of electrons from mitochondria and if improperly managed by cellular antioxidants is liable to induce the generation of reactive oxygen species (ROS). The ROS may cause oxidant damage to enzymes, nucleic acids, cytosolic and membrane proteins and the cellular lipids largely present in cell membranes. Carbon tetrachloride and halogenoalkanes are the most common chemical pollutants produced by industries and automobiles. These can be absorbed through the skin or ingested through water and they produce hepatotoxicity in virtually all species. The hepatotoxicity of halogenoalkanes involves reductive dehalogenation by cytochrome p450, of the hepatic microsomal mixed function oxygenase system to form trichloromethyl (CCl_3) and trichloromethylperoxy (CCl_3O_2) radicals (Brattin et al., 1985, Rosen and Rauckman, 1982). Although a consensus on direct proof for the generation of carbon tetrachloride free radicals has not yet been reached, indirect evidence for their microsomal formation is overwhelming

(Rosen and Rauckman, 1982). Carcinogenesis is thought to occur in two stages, first, the initiation stage, during which a physical, chemical or biological agent directly causes an irreversible alteration in the molecular structure of DNA and second, the promotion stage, during which the expression of genes that regulate cell growth and differentiation is altered. Experimental studies strongly suggest that free radicals are active in both the initiation and promotion stages of cancer, making it an important aetiological agent in carcinogenesis (Floyd, 1990).

Lipid peroxidation products

Polyunsaturated fatty acids are particularly vulnerable to free radical attack. The oxidative damage is termed lipid peroxidation and causes a reduction in membrane fluidity and permeability besides producing highly genotoxic and tumourigenic aldehydic lipid peroxidation products (Pandey et al., 1995, Shukla et al., 1994, Rosen and Rauckman, 1982). Linolenic acid (C18:2) and arachidonic acid (C20:4) contain a number of methylene-interrupted double bonds, which are particularly prone to hydrogen abstraction. This process can be autocatalytic. However, it can be halted by the action of free radical scavenger molecules (Chopra et al., 1989, Kirkpatrick and Lauer, 1986, Gutteridge et al., 1979). The NADPH-cytochrome p450 electron transport chain present in liver microsomes acts as an electron donor and promotes the generation of free radicals, thereby initiating lipid peroxidation (Slater and Sawyer, 1971b, Slater and Sawyer, 1971a). Of the various lipid peroxidation products, 4-hydroxynonenal (HNE) has a high neoplastic potential (Benedetti et al., 1980, Esterbauer et al., 1982, Esterbauer and Zollner, 1989,

Esterbauer et al., 1985). Shukla et al. have shown an increase in the concentration of HNE in gallbladder bile of patients with carcinoma of the gallbladder (Shukla et al., 1994). A further significant decrease in the concentration of polyunsaturated fatty acids (linolenic and arachidonic acid) in patients with gallstones has been demonstrated leading to speculation that increased lipid peroxidation along with non-functioning of the gallbladder, which causes HNE to be retained in the gallbladder for a long duration and in high quantities, may be responsible for the malignant conversion (Pandey and Shukla, 2000, Pandey et al., 1995, Pandey et al., 2000).

Molecular and genetic factors

In the past decade there has been an increased interest in identifying molecular and genetic factors responsible for cancer initiation, progression, proliferation and metastasis. Several factors have been studied in gallbladder cancer. Of these, much work pertains to the tumour suppressor gene p53 and K-ras oncogene. The p53 gene is one of the most frequently mutated genes in many cancers, including gallbladder cancer, where it appears to be an early event (Billo et al., 2000). These mutations are commonly observed on axons 5, 6, 7 and 8 (Fujii et al., 1996, Jonas et al., 1997). p53 mutations in gallbladder cancer have been demonstrated in carcinomas that arises in association with adenoma (along with APC gene mutations) and in those associated with pancratico-biliary malunion (Itoi et al., 1999, Itoi et al., 1996, Kanthan et al., 2000, Kim et al., 2001). The K-ras gene is one of the most commonly detected oncogenes in human cancers. K-ras gene mutations are commonly found in codon 12 in patients with gallbladder cancer (Hanada et

al., 1996). These mutations are also seen in tumours arising ab initio, in malignant transformation of premalignant lesions and in patients with pancreatico-biliary malformation (Hanada et al., 1999, Ajiki et al., 1996, Hanada et al., 1996, Iwase et al., 1997). Studies on allele-specific mutations and loss of heterozygosity (LOH) in gallbladder cancer have revealed LOH at the p53 locus in up to 90% of patients, at 19q (the site of the DCC gene) in 31%, at 5q (the APC gene) in 22% and at 13q (the Rb gene) in 22% of cases (Wistuba et al., 1995). Using micro-satellite markers a high frequency of allelic loss has been demonstrated at 2p (82%), 4p (50%), 4q (50%), 8q (60%), 9q (50%), 10p (50%), 14p (60%), 14q (50%), 16p (60%), 19p (50%), 21p (50%) and Xp (67%) (Nakayama et al., 2001). However, most of these studies are retrospective on archival tissue with small samples and hence no significant conclusions can be drawn from these studies.

The aetiology of gallbladder cancer remains far from clear and none of the above risk factors has been demonstrated to have a direct cause and effect relationship. However, it is fair to say that the disease is probably multi-factorial and more research is continuing to look at this difficult disease.

Pancreatic & periampullary cancer

The causes of pancreatic and periampullary cancer are not known. However, a variety of risk factors have been identified.

Smoking

The risk factor most consistently identified is cigarette smoking, which may account for approximately 25–30% of cases of pancreatic adenocarcinoma. Relative risks for pancreatic cancer generally ranged between 1.5 and 3 for

current smokers versus non-smokers (Silverman et al., 1994). A dose-response relationship has been reported by some studies with increasing dose and duration of smoking (Lin et al., 2002).

Familial pancreatic cancer

Five to ten percent of pancreatic cancers are hereditary in nature. Three to five fold higher risk of developing pancreatic cancer is reported in individuals with a close relative with the same disease. No single pancreatic cancer gene has been identified but a number of inherited cancer syndromes have been associated with higher number of pancreatic cancer. These syndromes include familial breast cancer (BRCA2 mutation), familial atypical multiple mole melanoma syndrome (FAMMM), hereditary pancreatitis, hereditary non-polyposis colorectal cancer (HNPCC) and Peutz-Jeghers Syndrome (Lal et al., 2000, Kern et al., 2002).

Other factors

Various other factors, including diet (high fat and protein, low fruit and vegetable intake), coffee consumption, alcohol, occupation, and the effects of other diseases such as diabetes mellitus, pernicious anaemia, chronic pancreatitis, cholelithiasis, and previous gastric surgery, have also been studied in detail. Of these, only in chronic pancreatitis and adult onset diabetes of less than two years' duration does there seem to be clear evidence of an increased risk of pancreatic cancer (Everhart and Wright, 1995, Lowenfels et al., 1993). Chronic pancreatitis is associated with an increased risk of cancer of the order of 5–15-fold. Periampullary cancers can be broadly considered as those tumours arising out of or within 2 cm of the

papilla of Vater and include ampullary, pancreatic, bile duct, and duodenal cancer. There is a high incidence of these tumours in patients with familial adenomatous polyposis (FAP). The median interval between colectomy for FAP and the development of upper gastrointestinal cancer is 22 years and cancer is often preceded by ampullary or duodenal adenoma or arises in an adenoma.

Molecular pathogenesis of pancreatobiliary malignancy

A number of mutations in oncogenes and tumour suppressor genes have been identified in cholangiocarcinoma, suggesting the cancer may arise secondary to cellular damage and consequent DNA injury (Berthiaume and Wands, 2004). Several studies have shown abnormal expression of the K-*ras* oncogene in 21–100% of cases and the p53 tumour suppressor gene in up to 37% of archival specimens in the largest studies (Nehls et al., 2004). These genetic alterations are associated with a more aggressive phenotype in this cancer (Isa et al., 2002, Nehls et al., 2004). K-*ras* and p53 mutations have also been identified in bile and pancreatic juice of affected patients (Nehls et al., 2004, Wang et al., 2002). Neither K-*ras* nor p53 mutational analysis have been shown to be superior to conventional cytopathology in the diagnosis of pancreaticobiliary tumours, but combined analysis of both can increase sensitivity in tissue biopsy and bile specimens (Itoi et al., 1999, Nehls et al., 2004, Wang et al., 2002). Increased expression of c-*met* and c-erbB-2 proto-oncogenes has been shown and they have been suggested to participate in the metastatic transformation of intrahepatic tumour (Aishima et al., 2002). Over expression of the protooncogene *Bcl*-2 has been reported to reduce apoptosis in cholangiocarcinoma cell-lines (Harnois et al., 1997).

Although human cholangiocarcinoma does not express Bcl-2, other antiapoptotic proteins, mcl-1 and Bcl-xl, are expressed. Point mutations leading to promotor methylation of cell cycle regulators p16INK4a and p14ARF have also been identified in cholangiocarcinoma related to primary sclerosing cholangitis (Khan et al., 2005). Other potential molecular markers for biliary malignancy are currently under investigation. These include growth factors and the occurrence of DNA aneuploidy, nuclear morphometry, and loss of heterozygosity of microsatellite markers (Itoi et al., 1999).

Mechanism of carcinogenesis

In recent years considerable progress has been made in understanding the pathogenesis of cholangiocarcinoma. Development of cholangiocarcinoma is probably a multi-step process involving the interaction between environmental and genetic factors. Malignant transformation of biliary epithelium occurs in an environment of chronic biliary inflammation and most of the environmental risk factors for cholangiocarcinoma cause chronic biliary irritation leading to inflammation. Several potential genetic changes and their interaction with environmental factors are summarised in Figure 1.6 (Berthiaume and Wands, 2004, Khan et al., 2005). In a recent review, Khan et al proposed that genetic polymorphisms in the cytochrome P450 enzymes or in the bile salt transport proteins could lead to alterations in the efficiency with which environmental toxins (xenobiotics) are handled by the liver (Khan et al., 2005). The development of cholangiocarcinoma probably needs a "second hit" to activate such xenobiotics and to expose the cholangiocytes to damage. Such secondary hits include chronic inflammation, viral hepatitis, worm infestation and recurrent cholangitis.

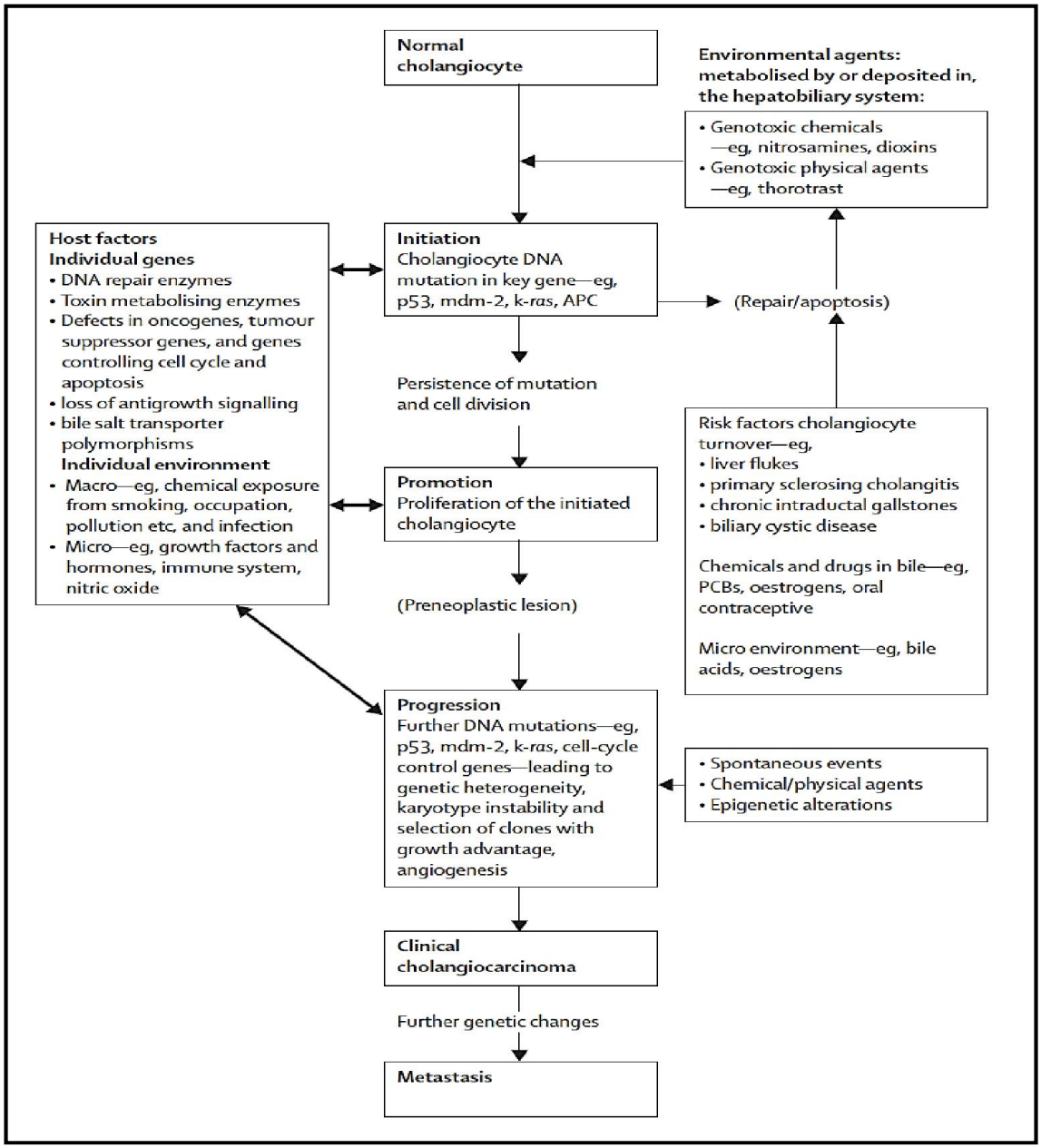

Figure 1.6: Proposed model for carcinogenesis in cholangiocarcinoma showing interaction between environmental factors and host genetics (Khan et al., 2005).

Environmental pollution, heavy metals and pancreato-biliary malignancy

Metals are natural elements that have been extracted from the earth and used for human industry and products for thousands of years but they also form a major category of environmental pollutants. Metals are notable for

their wide environmental dispersion, their tendency to accumulate in selected tissues of the human body and their potential to be toxic at a very minor level of exposure. Some metals, such as copper or iron, are essential to life and play a very important role in the functioning of critical enzyme systems. Other metals are xenobiotics, i.e, they have no useful role in human biology and worse are the metals like lead or mercury which may be toxic at even trace exposure. One reflection of the importance of metals relative to other potential environmental hazards is their ranking by the U.S. Agency for Toxic Substances and Disease Registry (ATSDR), which lists all hazards present in toxic waste sites according to their prevalence and the severity of their toxicity. The first, second, third and sixth hazards on the list are heavy metals: lead, mercury, arsenic and cadmium, respectively.

Exposure to metals can occur through various routes. They van be inhaled as dust or fumes or as vapour. They can also be ingested through food or drink as contaminants. The amount that is actually absorbed from respiratory or digestive tract is variable and depends on the chemical form of the metal and the age and physical status of the individual. Once absorbed, metals are distributed in tissues and organs excreted primarily through kidneys and digestive tract, but they tend to persist in some storage sites, like the liver, bones and the kidneys, for several years.

Acute and high dose toxicity to the metals usually involves the brain or the kidneys and produces generalized symptoms to start with, which makes their diagnosis quite difficult. More worrying is the long term low dose exposure, which may result in chronic illnesses like hypertension with exposure to lead

and renal impairment with cadmium and the potential of some heavy metals to be carcinogenic e.g. arsenic and cadmium.

A wide variety of metals including copper, lead, cadmium, iron, molybdenum, chromium, silicon and silver is reported in increasingly high quantities in people with cancer (Gupta et al., 1993, Delves et al., 1973). Inutsuka and Araki reported an elevated copper/zinc ratio in malignancy, including one case of carcinoma of the gallbladder and a further increase in advanced disease especially with liver metastasis (Inutsuka and Araki, 1978). There are reports of high cadmium concentration in renal and hepatic tissues of patients with bronchogenic carcinoma. There are authors who attribute this cadmium toxicity to its role as an antimetabolite of zinc. Several authors reported in the past that cadmium is a potent uncoupler of oxidative phosphorylation in vitro and that defective respiration is characteristic of tumour cells (Balo and Banga, 1957, Bradley et al., 1956). High levels of heavy metals in the bile of patients with carcinoma of the gallbladder have been reported (Shukla et al., 1998b). Metallothioneins (MTs) are a group of closely related low-molecular-weight proteins with a high affinity for the metals of groups I and IIB of the periodic table (Mulder et al., 1992). High levels of endogenous MTs bound to copper are observed in mammalian liver during gestation and in the early postnatal period (Bremner et al., 1977). The exact role of MTs is not yet clear, however, it is thought to point towards intercellular storage of essential metals (Webb and Cain, 1982). MT synthesis can be induced by the injection of certain metals (Onosaka et al., 1986). Increased MT expression has been demonstrated in patients with

carcinoma of the gallbladder compared to control patients and patients with cholecystitis (Shukla et al., 1998a). This suggests a possible role of heavy metal toxicity in gallbladder carcinogenesis and also as cadmium replaces copper from endogenous MT, the increased concentration of cadmium may give rise to an increased concentration of copper in the hepatic tissue and hence an altered copper/zinc ratio, as has been observed by various workers (Delves et al., 1973, Gupta et al., 1993).

In a meta-analysis of environmental studies looking at occupational exposures and pancreatic cancer, Ojajärvi et al concluded that about 20-25% of pancreatic cancer are related to smoking and about 15-20% are due to occupational and environmental pollutants in a typical “western” population (Ojajarvi et al., 2000). Exposure to nickel and chromium compounds had a relation to development of pancreatic cancer. Recently, a lot of interest has been focused on association of cadmium with pancreatic cancer. The strongest suspicion of an association between cadmium exposure and pancreatic cancer has been reported in Louisiana in the United States (Blot et al., 1978, Lemus et al., 1996). Industrial activity along the Mississippi River has led to an accumulation of contaminants in southern Louisiana (Mielke et al., 2000). Seafood and rice are popular food items in the local diet. Both rice and fish harvested from cadmium-polluted areas may contain high levels of cadmium (Ikeda, 1992). A case–control study in Louisiana showed a significantly increased risk for pancreatic cancer associated with rice consumption among the local population, with a dose response relationship (Falk et al., 1988, Falk et al., 1990). A more recent study published looking at

a population in the East Nile delta region in Egypt shows a strong correlation between cadmium exposure and development of pancreatic cancer (Kriegel et al., 2006). Schwartz and Reis in 2000 introduced a cadmium hypothesis for development of pancreatic cancer (Schwartz and Reis, 2000). The four commonly recognized risk factors for pancreatic cancer were interpreted with their association to cadmium exposure as in the following table (Table 1.4).

Risk factor	Explanation by cadmium hypothesis
Age	Cadmium accumulates in the pancreas with age.
Smoking	Cadmium is a contaminant of cigarettes. The pancreas of smokers contains twice the amount of cadmium as the pancreas of nonsmokers
Geography	Increased rates in Louisiana are consistent with cadmium pollution. International mortality rates are positively correlated with dietary cadmium.
Occupation	Occupations with high exposure to cadmium show increased mortality from pancreatic cancer

Table 1.4: Risk factors for pancreatic cancer and their interpretation by the cadmium hypothesis (Schwartz and Reis, 2000).

The evidence from research in areas with high environmental exposure to heavy metals quite strongly favours a relation between environmental pollution and pancreatobiliary malignancy. However, very little epidemiological research has taken place in areas, which do not seem to have major problems with environmental heavy metal exposure. The incidence of pancreato-biliary malignancy is also increasing in such areas and, therefore, it will be interesting to see whether environmental factors

have a significant correlation with increased incidence of pancreatobiliary malignancy in these regions.

An interesting study emerged from an area of high environmental pollution and high incidence of gallbladder cancer in the Northern Indian state of Uttar Pradesh. Shukla et al in a study published in 1998 demonstrated a relation between high level of heavy metals in the bile and gallbladder cancer. The study looked at a population in Northern India where there is significant environmental pollution with heavy metals. Bile samples from 38 patients with gallbladder cancer were compared with 58 patients with benign gallstones in a case control study (Figure 1.7).

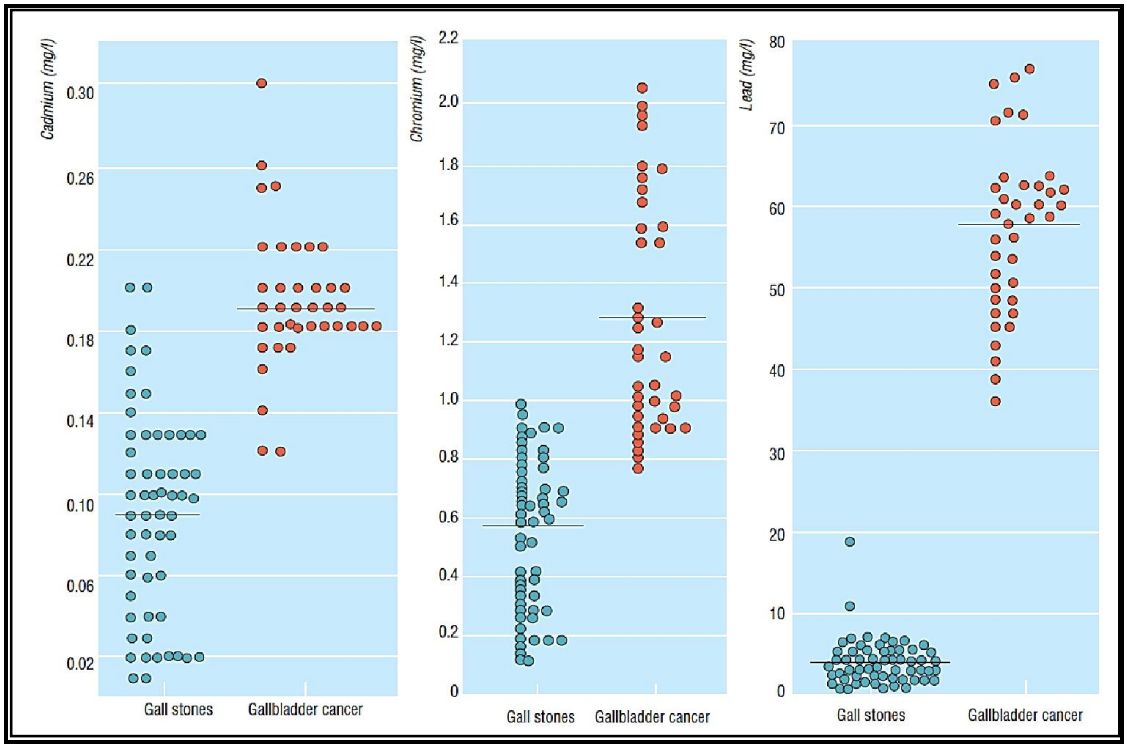

Figure 1.7: Cadmium, chromium and lead concentration in patients with gallstones and gallbladder cancer (Shukla et al., 1998b).

The figure shows the mean biliary concentrations of cadmium, chromium and lead are significantly higher in patients with gallbladder cancer than those with gallstones. This indicates a possible relation between environmental exposure to these metals and development of biliary cancer, although a direct cause and effect relationship cannot be established.

In the North-East of England, environmental pollution is not as much a problem as in Northern India. However, this part of the country has been traditionally an area of heavy industry including mining and shipbuilding. Although these traditional industries are no longer a major contributor to the local economy, this region still has pockets of heavy industry, including chemical industry, especially in the southern part of this region in Teesside. To investigate the possibility of local air pollution contributing to health problems, a study in 1998 by the Department of Epidemiology and Public Health, University of Newcastle, looked at different areas of Teesside according to proximity to industry and also compared it with the neighbouring metropolitan population in Sunderland. There was no significant difference in health problems, although there was a trend towards increased incidence of lung cancer amongst the population closer to industry, but this trend was not statistically significant (Bhopal et al., 1998). The same group studied the relation of lung cancer, proximity to industry and social deprivation in Teesside and found an increased incidence of lung cancer related mortality in women under 75 years of age living in close proximity to industry (Pless-Mulloli et al., 1998). This group continued to work on the same theme in the North East of England and looked at the duration of exposure to

environmental pollution in populations living close to heavy industry and reported a modestly increased risk of lung cancer (Edwards et al., 2006).

Similar projects looking at the possible relationship of biliary and pancreatic cancer with heavy metal exposure in this country have not been done. Investigations done abroad on areas of high environmental exposure to heavy metals provide interesting data for carrying out similar project in the UK (Kriegel et al., 2006, Shukla et al., 1998b).

The present project was based on the model from India published by Shukla et al in 1998. This was a simple model where patients with gallbladder cancer had a sample of bile collected during surgery and different heavy metals levels in the bile were measured. Bile sample was also collected from a group of patients with benign gallstone disease and the levels of each heavy metal in these 2 groups were compared. However, the project in India was carried out in a region of heavy industrial pollution with obvious exposure to heavy metals for the population living in those areas. Although areas in the North-East of England have heavy industry, the degree of pollution is not as high as experienced in India. Epidemiological projects done locally in the North-East of England on environmental pollution and lung cancer supports similar project to look at the possible relation of heavy metals and pancreato-biliary malignancy (Bhopal et al., 1998).

Chapter 2

Aim of the Study

2. Aim of the Study

Cancer is a serious health problem, both in the UK and across the North-East of England. There were 243,400 new cases of cancer in England in 2006, of those 13,600 were in the North East. There were 7,500 cancer related deaths in the North-East of England in the same year. Between 2001 to 2005, the incidence of all cancers in England (excluding non-melanoma skin cancers) was 380.0 per 100,000 population, and the incidence was 411.6 per 100,000 in the North-East of England over the same period. The mortality rate for all cancers is also higher in the North East compared to England (214.8 compared to 187.7 per 100,000 population respectively). The five-year survival rate for all cancers is 48.4% across England compared to 43.2% in the North East (North East Public Health Observatory, (www.nepho.org.uk).

The above data highlights the need for improvement in cancer related services as well as cancer research in the North East of England. There has been a major move towards centralization of cancer services in this region in the past decade led by the Northern Cancer Network. Hepato-biliary and pancreatic cancer surgery has been centralized to the tertiary referral centre at Freeman Hospital, Newcastle upon Tyne. The present project was conducted in the Freeman Hospital and Department of Surgery and Department of Agriculture, University of Newcastle upon Tyne. Being the tertiary referral centre for the whole region, all patients with suspected

hepato-biliary and pancreatic cancers are referred to the Freeman Hospital and that helped in identifying and recruiting patients for the study.

The main aims of the study were as follows:

1. Identify and recruit patients with suspected malignant biliary obstruction and benign biliary disease (e.g. gallstone).

2. Collect bile samples at first intervention in the biliary tree.

3. Measure the concentration of different heavy metals (Copper, Zinc, Lead, Chromium, Nickel & Cadmium) in the bile samples collected from patients with biliary cancers and a control group with benign biliary.

4. Compare the 2 groups using standard statistical methods and analyze any significant difference in any of the metal levels in the 2 groups.

5. Possible correlation of results with geological distribution of the diseases

Although areas in the North-East of England have heavy industry, the degree of pollution is not as high when compared to previous similar studies from India or Egypt and we were concerned that there may not be measurable levels of heavy metals in the bile of local population in the North East of England.

In view of the nature of the study and the uncertainty of the logistics, it was decided to perform a feasibility study before embarking on a larger project. The aims of the pilot study were as follows:

1. Collect bile sample from a small group of patients with suspected or proven hepato-biliary and pancreatic cancer and also from a control group of patients with gallstone disease.

2. Examine the logistics of collecting the samples and find a method to make sure that all patients with suspected biliary cancer can be approached for the study.

3. Carry out preliminary analysis of the bile samples in the laboratory in order to standardize the method of bile sample analysis.

4. Carry out statistical analysis to compare groups in order to find the exact statistical tests to be used for the final study

Chapter 3

Material & Methods

3. Materials & Methods

Subject and study design

The present study was designed carefully with emphasis on including every single patient who was referred to the Freeman Hospital with suspected biliary cancer. Not all patients get referred with a definite diagnosis of biliary cancer. The majority of the patients with suspected cancer of the bile duct, gall bladder or pancreas have been investigated by at least an ultrasound scan or a computerized tomographic (CT) scan and there may be evidence of malignancy on these imaging modalities. One common presentation in most of these patients is the presence of jaundice. This is caused by the partial or complete obstruction of the bile duct by the tumour. It is often the first complaint of the patient in this group of cancers. Because of the consistency of the symptom, we felt that all patients presenting to the unit with obstructive jaundice would be approached for the study. Based on previous publications, it was planned for two groups of patients to be studied - patients with biliary tract cancers and a control group of patients with non-malignant hepatobiliary disorders such as gallstones.

Consent forms and patient information sheet were designed after approval of the local Joint Ethics Committee (Appendix I & II, Joint Ethics Committee approval reference 2001/33). Every patient approached for the study had the opportunity to go through the information sheet and discuss with the investigator before consenting to take part in the study. It was our priority to

ensure that bile samples be collected from the patients with minimum discomfort or inconvenience to the patients.

As part of their routine diagnostic procedure and treatment there were opportunities to obtain bile samples without additional risk to the patients. Patients with biliary tract cancers commonly undergo investigations and minimally invasive procedures or surgery, or both. Minimally invasive procedures are carried out both for investigation of malignant biliary obstruction as well as with a therapeutic intent to drain the biliary system by different types of stents to improve jaundice. Interventions include endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography (PTC). During these procedures the bile duct is accessed and bile samples can be aspirated. During surgery there is an opportunity for obtaining bile samples from both gall bladder and the bile duct. Since the gallbladder concentrates the bile it receives from the liver, the heavy metal concentration in the bile obtained from the gallbladder may be expected to be different from hepatic bile. Bile was, therefore, collected from both sources whenever possible to look for any significant difference.

Patients with symptomatic gallstones or benign biliary obstruction were selected as controls. They may require surgery and some patients will require ERCP for diagnostic or therapeutic purposes. Bile samples can, therefore, be obtained from each group of patients without any additional procedure or added risk for the participants in the study.

Since obstruction to the bile duct causes jaundice, stagnation of bile or cholestasis may lead to changes in biliary heavy metal concentration, we proposed to carefully note the degree of biliary obstruction as shown by the liver function tests, including the bilirubin level at the time of collecting the bile to investigate the possible influence of biliary obstruction on the heavy metal concentration. A dataset of important patient variables and risk factors for the development of biliary tract cancers were established and recorded on a Microsoft Access Database created specifically for this project.

A direct comparison was made between the levels of heavy metals in the hepatic and gallbladder bile in all subjects. Heavy metal concentration was correlated with conventional markers of cholestasis severity such as serum bilirubin level. Biliary concentration of heavy metals in cancer patients was compared with controls and the association (if any) between biliary heavy metal concentrations and the presence of malignancy (corrected for the presence of cholestasis) assessed.

Bile Sample Analysis

Collection, storage and handling of bile samples

Patients were approached at the time of their attendance at the Freeman Hospital for their ERCP/PTC or before surgery. Hepatic bile was obtained in both groups during ERCP/PTC or during surgery. Gallbladder bile was obtained during operation in both groups. All samples were coded using a unique number and stored at the Research Unit freezer in the Freeman Hospital at - 84°C till they were extracted for analysis. When a suitable

number of samples were collected for analysis, they were transported to the laboratory of the Department of Agriculture, Newcastle University, for analysis. All the samples were defrosted and thawed before the analysis began.

As the samples were human products, a COSHH assessment was carried out and it was felt necessary that personnel handling these samples were vaccinated against Hepatitis B. Necessary precautions were taken to avoid biohazard and all unused samples and containers were destroyed by incineration.

There were concerns about whether there will be measurable levels of heavy metals in the bile of patients from the North-East of England. Similar projects looking at heavy metal concentrations in bile were all from areas with high environmental pollution and high metal concentration in serum or bile was expected. We, therefore, decided to set up a pilot project to ascertain if adequate level of metal was present in the bile samples. This will also give us the opportunity to run the full process of analysing the samples and address any problems that can arise during laboratory work.

Although the pilot project is part of the methods of this study, it is described in a separate chapter for ease of understanding and it also allows us integrate the data that emerges out of the pilot study with the main project.

Chapter 4

Pilot Study

4. Pilot Study

As mentioned earlier, we were keen to ascertain whether there were measurable levels of heavy metals in the bile of patients in the North-East of England before embarking on a bigger project. The pilot study would also give us the opportunity to standardize the methods of bile sample analysis, which has never been done in our laboratories before.

Standardisation of methodology – the initial samples

Crude bile samples obtained from patients were not suitable for analysis in the spectrometer directly. The bile samples needed pre-treatment before the quantitative analysis for heavy metals in spectrometer and it is standard practice to pre-treat crude samples in this way. Two methods of pre treatment were assessed – syringe filtration method and acid digestion method. The samples were split in half, allowing for at least 5 ml to be set aside for the acid digestion method and the rest of the sample was syringe filtered.

Syringe filtration method

The tubes containing the samples were inverted 10 times to fully mix the contents. This prevents analysing only a fraction of the sample and ensuring that this method and the acid digestion method could be compared. Each sample was syringe filtered until there was approximately 2 ml of filtrate, an adequate amount to test for four metals – lead, cadmium, zinc and copper in the spectrometer.

Originally the samples were forced through a 0.5 µm filter attached to the end of the syringe. Unfortunately these filters were blocked with mucus very quickly and up to 6 or 7 filters were needed for each sample. This was relatively expensive and labour intensive method. It was therefore proposed to use a prefilter as well as the 0.5 µm filter for each sample and the method was successful. The filtrate was collected in plastic tubes with plastic lids and taken to the spectrometer for analysis.

Acid digestion method

The tubes containing the samples were, as with the syringe filtration method, inverted 10 times to fully mix the contents. 5 ml of sample was pipetted into glass digesting tubes with plastic leads and 5 ml of concentrated nitric acid was added to them and the samples left overnight. As soon as the acid was added there was a visible reaction with the sample changing colour to a clear pinkish solution usually and there was some coagulation of organic materials in the sample. The following day the samples were heated up to 120°C over an 8-hour period in a Grant BT5 heating block to complete the digestion. After allowing them to cool overnight the samples were taken in plastic tubes for analysis in the spectrometer.

Spectrometric analysis

Both the syringe filtered and acid digested samples were subjected to spectrometric analysis. An ATI Unicom SolaaR 929 atomic absorption spectrometer was used with a deuterium background interference application for greater accuracy (Figure 4.1). Due to presence of large concentration of salts in the samples, the flame turned orange during analysis. It was prudent

to wait for the flame to lose the orange colour and therefore burn off all the salt before adding the next sample. The spectrometer was calibrated with standards before each session of sample analysis. Each sample was taken through the intake tube, which was placed in distilled water between each

Figure 4.1: ATI Unicom SolaaR 929 atomic absorption spectrometer

sample. The results for the syringe filtered samples were a direct measurement but with the acid digested samples the results needed to be doubled as the sample had been diluted by a factor of 2 by the acid.

Results of pilot study – first 25 samples

25 samples were analysed using both acid digestion and syringe filtration methods as part of the initial analysis. The results of these analyses were used to standardise the methods for subsequent samples. We have

compared the results of these 2 methods with the aim of choosing one of them as the standard method with the possibility that one of them will produce a higher or more measurable concentration of the metals in bile for subsequent analysis.

Acid digestion versus syringe filtration

<u>Zinc</u>

There is no significant difference between acid digestion and syringe filtration methods on analysis. The mean concentration of zinc is smaller in acid digestion method than in syringe filtration method, and the difference is not statistically significant (P=0.33, Mann-Whitney U) (Figure 4.2)

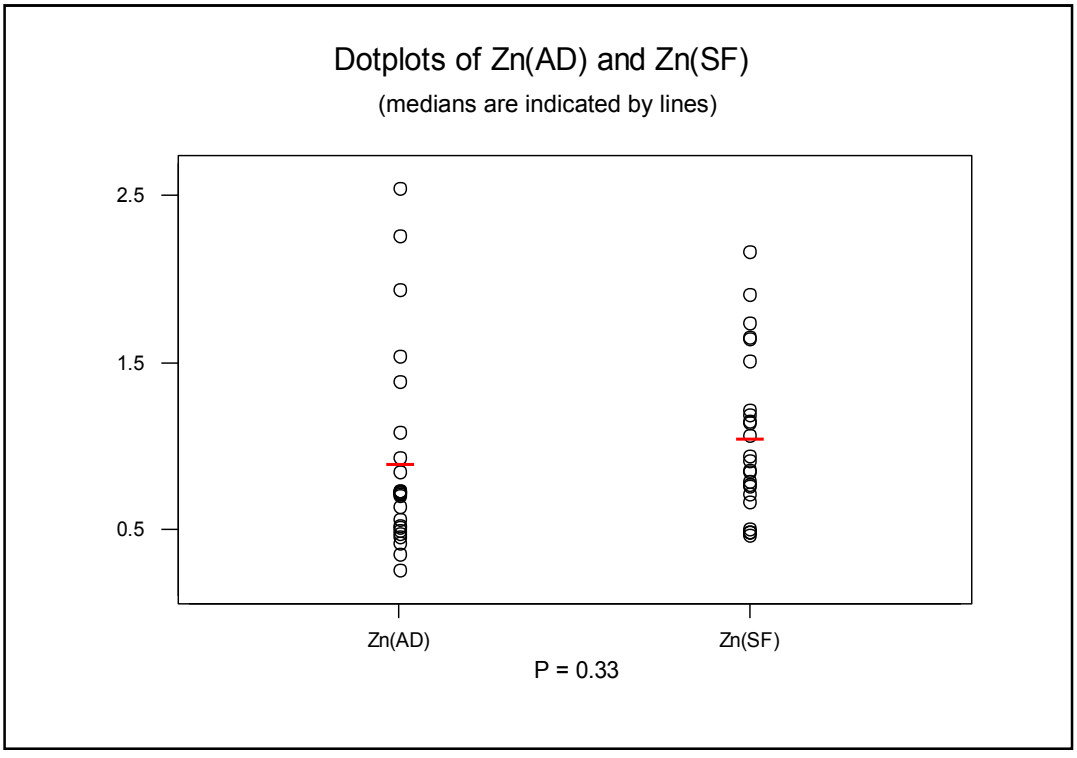

Figure 4.2: Comparison of acid digestion (AD) and syringe filtration (SF) method for concentration of zinc (Zn)

Cadmium

There was a large difference between the acid digested sample and the syringe-filtered samples. Acid digestion produced a much greater concentration of cadmium than syringe filtration. The difference between the median concentration of cadmium obtained by the two processes were statistically significant (P=<0.01) (Figure 4.3)

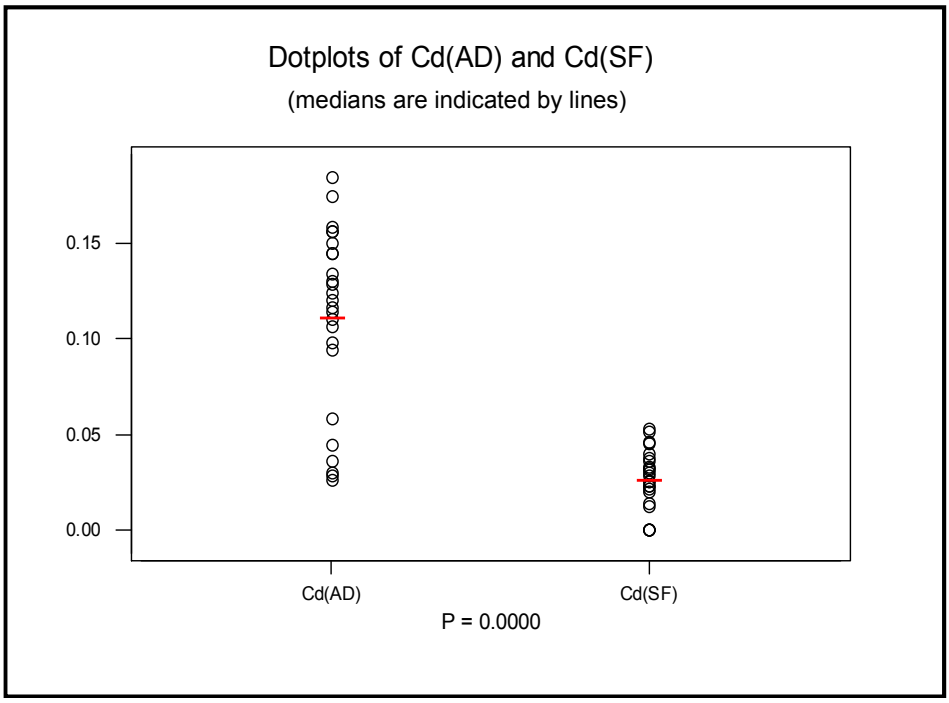

Figure 4.3: Comparison of acid digestion (AD) and syringe filtration (SF) method for concentration of cadmium (Cd)

Copper

Both sets of samples had similar mean values with no major difference. They both had a close grouping of results around the median and a few very large values. Going back at those samples we noticed that the samples had been first collected in bottles with a brass cap and as brass is an alloy of copper and zinc, we thought it may produce erroneous results. However, not all samples stored in bottles with brass caps had high copper levels. In order to

avoid this possible source of error, all samples subsequently had been stored in glass containers with plastic caps. As evident from the figure, there was no statistical significance in the difference of means between the two groups (P=0.31) (Figure 4.4)

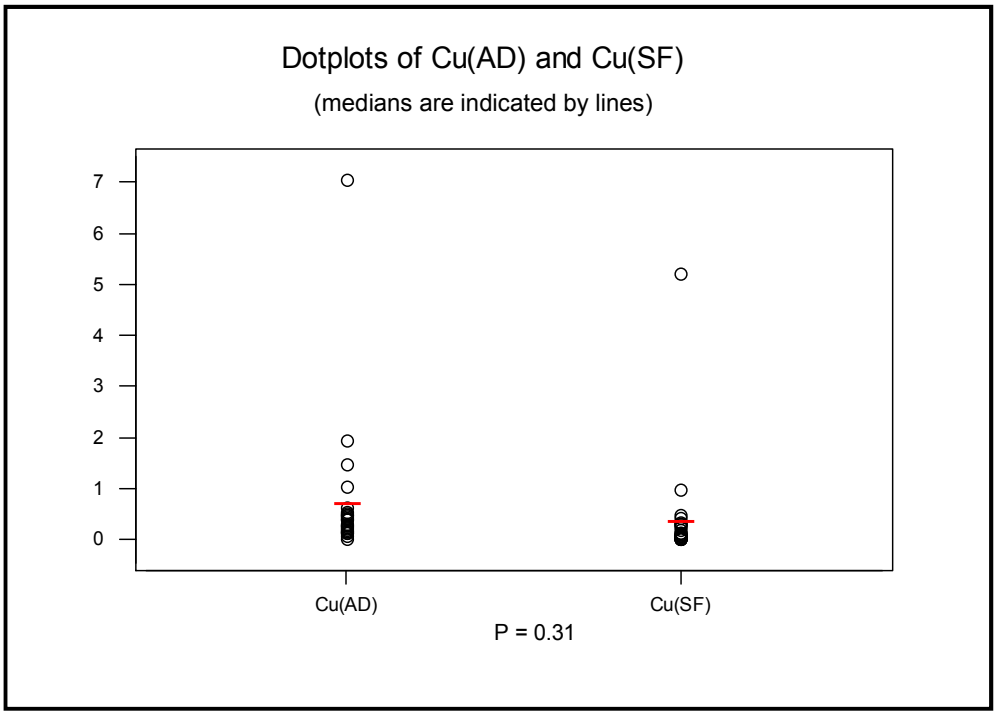

Figure 4.4: Comparison of acid digestion (AD) and syringe filtration (SF) method for concentration of copper (Cu)

Lead

There was no measurable concentration of lead in the syringe-filtered samples and there was measurable concentration in the acid digested samples. Therefore to analyse the samples for lead concentration, we could only use the acid digestion method.

The above finding for lead concentration and the significantly better results with cadmium meant that acid digestion would be the appropriate method for pre-treatment of bile samples before spectrometric analysis. After discussion

with agricultural scientists with vast experience of heavy metal assay for river water and soil samples where above methods of pre-treatment is commonly used, it was decided that acid digestion would be used as the standard procedure for pre treatment for the final analysis.

Comparison between benign and malignant

In the initial 25 samples, there were only 4 samples from the control group with benign biliary disease. The 2 groups were, therefore not balanced and direct comparison between the 2 groups was not appropriate. However, comparison was made for each metal between the 2 groups, none showing any significant result. A simple comparison of medians of the metal levels in the study group and the control group was done using non-parametric test (Mann-Whitney U). Lead and cadmium are the 2 metals that had the most difference in concentration between the 2 groups in the original study from India by Shukla et al. The results of these 2 metals are given below in the form of dotplots (Figure 4.5, Figure 4.6).

Lead

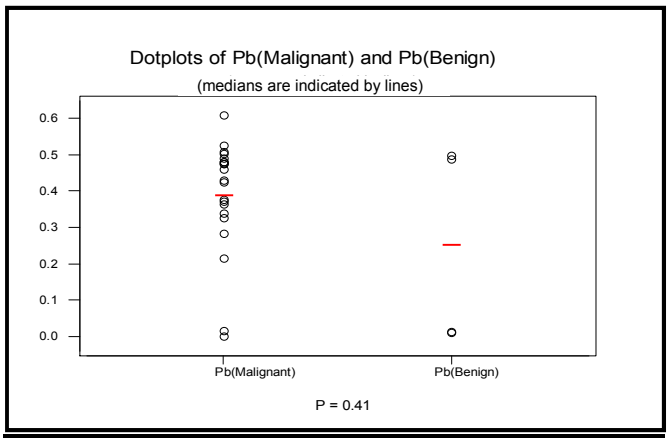

Figure 4.5: Comparison of median concentration of lead (Pb) in the bile samples from patients with benign and malignant diseases

Cadmium

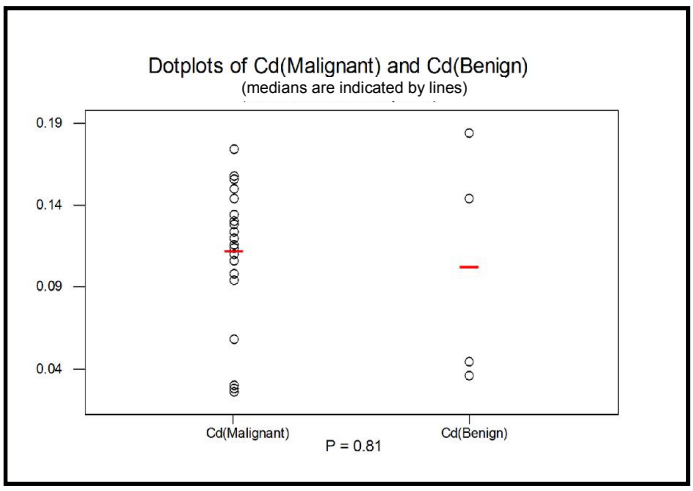

Figure 4.6: Comparison of median concentration of cadmium (Cd) in the bile samples from patients with benign and malignant diseases

A simple statistical analysis was also done to compare benign versus malignant samples from these initial 25 samples. Although this will be inadequately powered and the result of this analysis will be of no significance, it would help us to get an idea about the methods to be used for the final study. As evident from the above figures, there was no significant difference in the median level metals in the initial samples.

The pilot study was very useful in clarifying the aims that we set out with. It confirmed that there was measurable level of heavy metals in the bile samples collected from patients in this part of the world. We could finalize an efficient method of collecting the samples and storing them. The bile sample analysis in the laboratory could be standardized and with the supporting evidence from above, we decided to use acid-digestion method of pre-treatment as the standard method for the final project. We decided that data would be collected under the heading of individual metals and comparison

done between malignant and benign group. As we are dealing with a rather rare disease group, we decided to combine the results of the assays done in the pilot study by the acid digestion method with the results of the main study so that we can get adequate number in each group for comparison. For comparing different groups, the median rather than the mean was taken as the measure of central tendency in the final analysis as the results are not normally distributed and non-parametric tests have been used to compare them.

Chapter 5

Main Study

5. Main Study

Calculation of sample size

A calculation of sample size was done on the basis of the original study from India (Shukla et al., 1998b). The difference in mean concentration of lead in study and control group was very high (58.38 mg/l *vs* 3.99 mg/l) and a calculation based on this with a significance of 0.05 and a power of 90% demonstrated that approximately only 13 patients are required in each group for a significant result. Even calculation based on mean levels of cadmium, which had the least difference between the 2 groups (0.19 mg/l *vs* 0.09 mg/l), estimated approximately 27 patients in each group to demonstrate a significant result with same power. However, it is unlikely that such large differences would be seen in the UK and therefore it was proposed that all patients with biliary malignancy would be included in the study over a period of one year. The majority of patients with biliary cancers in the North of England are referred to our institution and it was felt that it would be possible to recruit at least 50 patients with biliary cancers and a similar number of patients with gallstone disease to act as control over a period of 12 months.

A significant difference in the concentration of heavy metals in the bile of these patients would be an important finding, particularly in the North of England, which is still an area of heavy industry. It would prompt further research into specific environmental pollutants and enable further collaboration with departments of occupational health, epidemiology and environment.

After completion of the pilot study, we continued collecting samples with the aim of collecting as many samples as possible to achieve at least 50 samples in each group. This number was agreed after calculation of sample size as described earlier.

Every patient attending Freeman Hospital with obstructive jaundice suspected to be due to a malignant biliary obstruction were approached for inclusion in the study.

In the subsequent analysis, all the samples were treated by acid digestion as described above and underwent spectrometric analysis. Nickel and chromium were analysed in the original study from India and these 2 metals were also included in the study at this stage. The plan was to make a direct comparison of mean concentration of each individual heavy metal between the study group (malignant pancreato-biliary disease) and controls (benign pancreato-biliary disease). This formed the basis of subsequent analysis with all the bile samples. As described above, we tried to collect bile sample from every patient with suspected malignant biliary obstruction. The individual samples were formally allotted to the study group (malignant pancreato-biliary disease) only after confirmation of diagnosis subsequently by positive histology, cytology or overwhelming radiological evidence of malignancy with raised tumour marker (CA19-9). If the radiological evidence or level of tumour marker were equivocal in a patient with biliary obstruction, we excluded such samples from the study.

We recognized at this stage that the study group comprising of patients with malignant biliary obstruction might not be a homogenous group of patients.

Patients with pancreatic cancer and biliary cancer (bile duct cholangiocarcinoma or gallbladder cancer) will both present with malignant biliary obstruction but they suffer from completely different diseases and clinically, pathologically and as far as long term prognosis is concerned, they behave in very different ways. It was felt that once all the data were collected, it might be worthwhile looking at each individual subgroup causing malignant biliary obstruction to see if that influences the result.

In order to facilitate data collection, we decided to create a database using Microsoft Access© software. It was a simple database where data entry could be done using the form view (Figure 5.1). Usual demographic data was collected. Attempt was made to collect data about smoking habits, possibility of heavy metal exposure and duration of exposure. Liver function test results were documented to see if presence of biliary obstruction had any effect on the concentration of biliary heavy metals. The source of the bile sample, i.e gallbladder or common bile duct, was also documented. The bile sample was collected during the first intervention in the bile duct, which could be endoscopic procedures like ERCP or radiological procedures like PTC. In either case the bile sample will be from the bile duct. On the other hand, the bile sample collected during surgery for benign disease like gallstone will be from gallbladder only. In patients undergoing resection for malignant disease, bile could be collected from both sources in order to have a set of samples where the bile was collected from two sources in the same patient and this subset would help us to see if there was any influence of the source of bile on the heavy metal concentration.

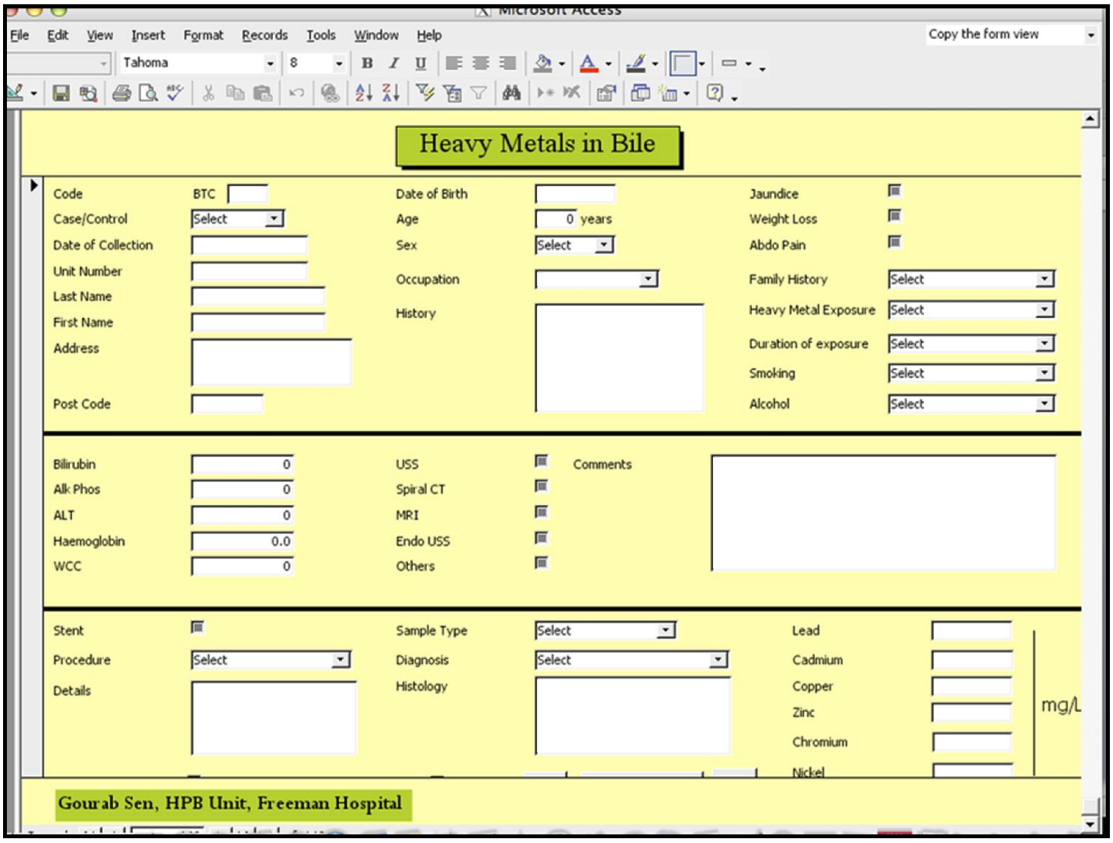

Figure 5.1: Data entry form for present study (Microsoft Access©)

The data from this database could be directly transferred to any statistical software for analysis. We used SPSS Version 16© (SPSS Inc, USA) for all the statistical analysis and the results output from the software is directly incorporated into the result section of this document without any modification. The charts and diagrams were produced by the same software using the same data, thereby minimizing the chance of error from using multiple softwares.

Results of main study

The results for the study were analyzed with the aim of comparing the mean concentration of each individual metal amongst the different study groups i.e. biliary cancer and benign biliary disease. As discussed in the methods of this

study, the samples of bile were collected from every patient with presumed malignant biliary obstruction, as it was not possible to have a definite histological or cytological diagnosis in all cases before their first intervention to the biliary tree. This was the only way of making sure that all the patients with possible biliary cancer could be potentially included in the study group. Once the final diagnosis was established, by cytology or histology or imaging with supporting elevated tumour marker, the individual samples were then allotted to the different study groups.

We followed a definite plan for the analysis of the final results.

1. Analyze the demographics of the patients from whom the samples were obtained
2. To ascertain whether the source of bile, either from common bile duct or gallbladder, has any impact on the metal concentration.
3. To ascertain whether the presence of biliary obstruction has any impact on metal concentration
4. Calculate the mean concentration of each individual metals and compare their levels between the malignant groups and the benign control group

One hundred and sixty two patients were approached for taking part in the study over a period of 20 months. This included all the patients with presumed malignant biliary obstruction and the patients with benign biliary disease (e.g. gallstones) as the control group. One hundred and twenty nine patients agreed to take part in the study and ultimately 107 patients were

recruited after excluding the patients who already had metal stent placed in the biliary tree before referral, or already started palliative chemotherapy or radiotherapy before inclusion.

.A total of 140 samples were collected from the 107 patients recruited for the study and in 33 patients, bile was collected from 2 sources (CBD and GB). The table below summarises the sources and number of bile samples used for final analysis (Table 5.1).

After excluding cases for inadequate samples and pathological diagnosis not directly related to biliary epithelial tumours (e.g. neuroendocrine tumours), we had a total of 127 samples to analyze. Specific statistical tests used for comparing groups are mentioned for specific analysis and results.

- Total number of patients approached for the study 162
- Total number of patients recruited 107
- Total number of samples of bile 140
- 33 patients had samples from 2 sources (CBD or GB)
- 74 patients had sample from a single source, either CBD or GB
- 3 excluded due to histological diagnosis – carcinoid/neuroendocrine tumour
- 1 sample from above 3 had inadequate volume for analysis
- 10 more samples excluded for inadequate volume
- Total number of samples analyzed {140 – (10+3)} = 127

Table 5.1: Details of analyzed samples

Age and Sex distribution

The total number of 127 samples came from 98 patients. The median age of the study population was 65.00 years (Standard deviation 12.87years, range 20 - 82 years). The age distribution is shown in the table (Table 5.2) and histogram below (Figure 5.2).

Total Number of Patients	98
Mean Age in years	62.18
Std. Error of Mean	1.300
Median	65.00
Std. Deviation	12.867
Minimum in years	20
Maximum in years	82

Table 5.2: Age distribution of the study population

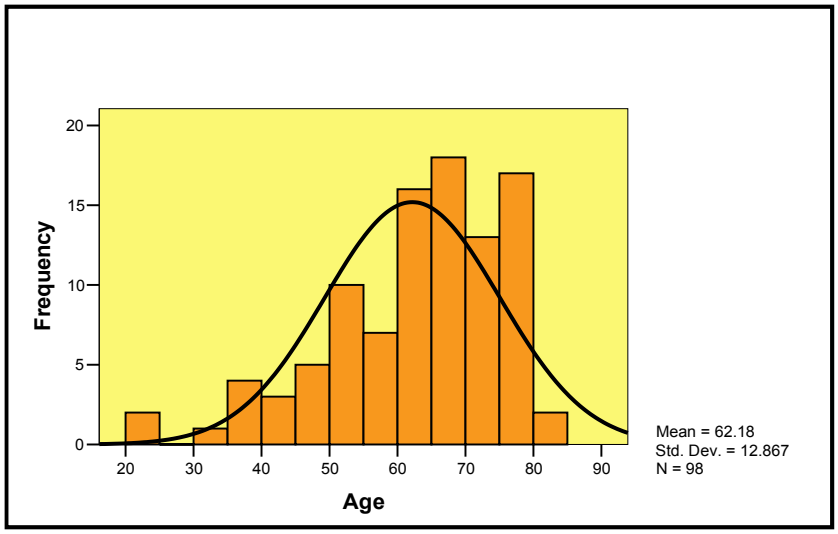

Figure 5.2: Histogram of the age distribution of the study population

The whole cohort of patients comprised of 55 female and 43 male patients. The sex distribution is depicted in the figure below (Figure 5.3).

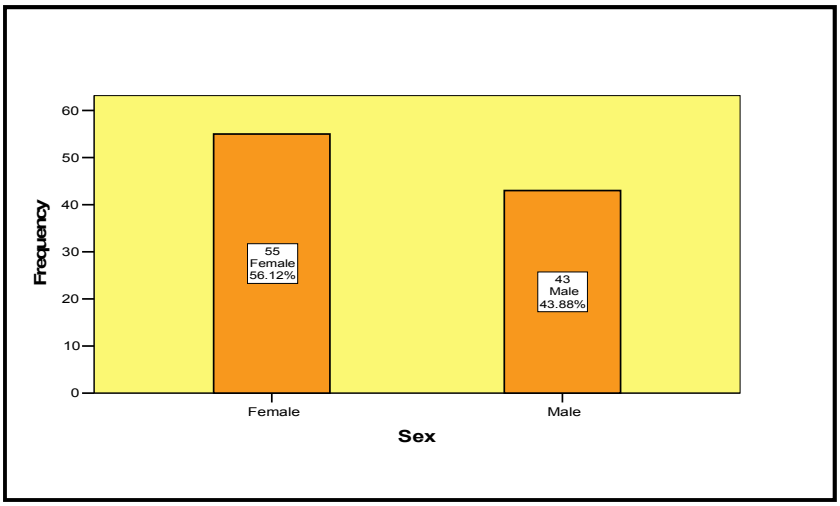

Figure 5.3: Sex distribution of all the patients

According to the aims of the study, analysis was carried out to compare the individual metal levels between the groups of patients with biliary malignancy versus those with benign biliary disease. As explained earlier, all patients with assumed malignant biliary obstruction were initially placed in the study group. However, we accepted that this study group will not be a homogenous group, as there will be different causes of malignant biliary obstruction. It was decided that to include a patient in this study, there must be either a histological or cytological diagnosis either by brush cytology from the bile duct, histology from endoscopic specimen or histology of resected specimens, or there must be overwhelming evidence of malignant process on imaging or endoscopic investigation along with a raised tumour marker (CA19-9) to support the diagnosis of malignancy. With this inclusion criteria, 68 patients (69.4%) had a positive histology or cytology to confirm malignancy and 30 patients (30.6%) were diagnosed on the basis of imaging and tumour markers. When the final diagnosis was available, it became apparent that the samples belong to essentially 4 groups of patients (Figure

5.4). Pancreatic cancer is a distinctly different disease both clinically and prognostically from either biliary or periampullary cancer and therefore we felt that for the purpose of discussion and analysis of results, these different types of cancers should be considered separately and not as a broad group of malignant diseases.

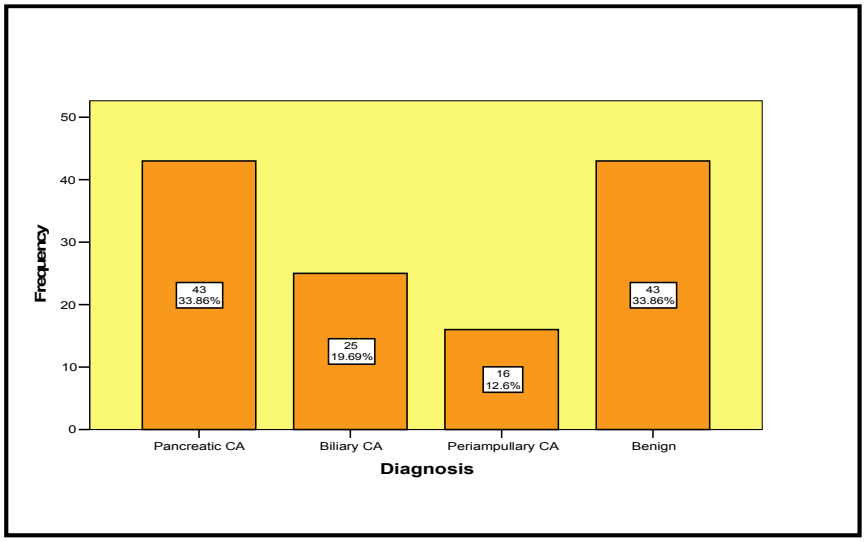

Figure 5.4: Histological diagnosis (CA = Carcinoma)

Age and sex distribution according to diagnosis

Once it was established that the analysis would be based on these four study groups, statistical analysis was done to assess the age and sex distribution of each group to see if there was any difference between the groups.

Diagnosis	N	Median	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Pancreatic Carcinoma	43	66.00	9.587	61.61	67.51	33	82
Biliary Carcinoma	25	71.00	7.826	65.85	72.31	51	81
Benign	43	57.00	14.137	51.30	60.00	20	78
Periampullary Carcinoma	16	69.50	7.856	63.44	71.81	54	77
Total	127	65.00	12.064	60.70	64.94	20	82

Table 5.3: Age distribution of individual groups of patients

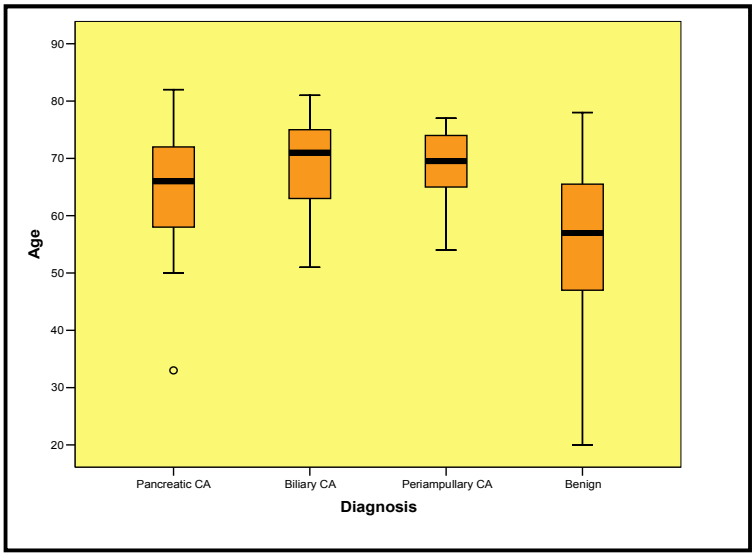

Figure 5.5: Age distribution of individual groups of patients

Descriptive statistics of individual groups and their mean age (Table 5.3) is shown above along with a box plot showing the mean (Figure 5.5). As would be clinically expected, the mean age of the benign group (55.65 yrs, standard deviation 14.14, range 20 – 78 years) appeared to be lower than all the 3 other groups with malignant disease. To test whether the difference in the mean was significant or not, they were compared using one-way ANOVA and the result suggests that there is significant difference between the groups ($p<0.0001$). It is to be noted, this test does not compare one particular group from another, but compares differences between all the 4 groups. The significant difference in means suggests the groups are not age-matched and this is likely to be contributed mostly by the lower mean age of the benign group.

Sex distribution according to diagnosis was analyzed using a similar strategy. The sex versus diagnosis cross tabulation is shown below (Figure 5.6).

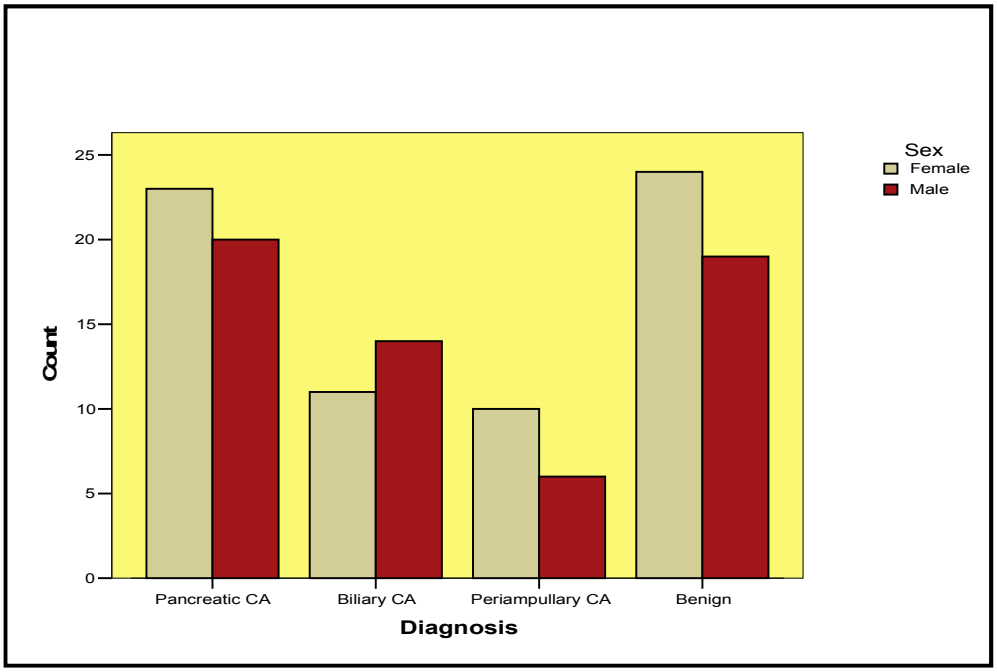

Figure 5.6: Sex distribution according to diagnosis

To test if there is any difference in the sex distribution amongst the different groups, a Chi-square test was done on the cross table and the result is given below (Table 5.4).

	Value	Df	Significance (2-sided)
Pearson Chi-Square	1.521	3	0.678
Likelihood Ratio	1.525	3	0.676
N of Valid Cases	127		

Table 5.4: Sex distribution vs. diagnosis (Chi-Square Test)

As the table suggests, there is no significant difference between groups in their sex distribution (p=0.68). The groups are reasonably sex matched.

Regional variation

The main source of heavy metals in the body is from environmental exposure. All the samples were from patients in the North-East of England, which has significant variation in level of industrialization. Although the level

of exposure will depend on a number of factors including duration of residence near possible sources, occupation etc, a base line analysis was done to see if there was a difference in the groups regarding which part of the North East of England they came from. The region was divided into North and South on the basis of the postcodes as the southern part has more heavy industry than the north. The results are given in Table 5.5 and Figure 5.7 below.

Regional distribution of patients		Diagnosis				Total
		Pancreatic CA	Biliary CA	Benign	Periampullary CA	
Post Code	North	23	19	31	9	82
	South	20	6	12	7	45
Total		43	25	43	16	127

Table 5.5**: Distribution of patients from the North and South of the region depending on the post code**

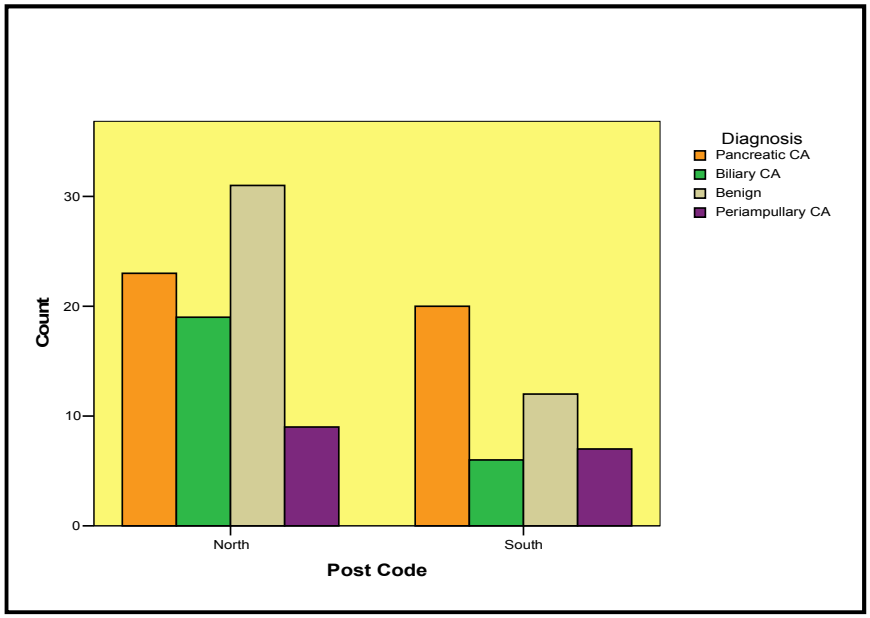

Figure 5.7: Distribution of patients into North and South of the region according to diagnosis

From the bar chart there appears to be a difference in the number of benign specimens with samples from the north being more than the south. This is

expected as the benign diseases from the south of the region are more likely to be treated locally and not referred to a tertiary centre. However, a Chi square test on the cross table did not show any significant difference in distribution of the samples originating from the north or south of the region (p=0.15)

Variation in levels of obstruction to the biliary tree in different groups

Some of the patients, especially those in the groups with malignant disease, presented with jaundice secondary to bile duct obstruction. There may be a potential influence of presence of significant number of obstructed patient in any particular group. A serum bilirubin level of over 35 µmol/L was taken as a cut off and all patients with serum bilirubin higher than that was considered to be having an obstructed biliary system.

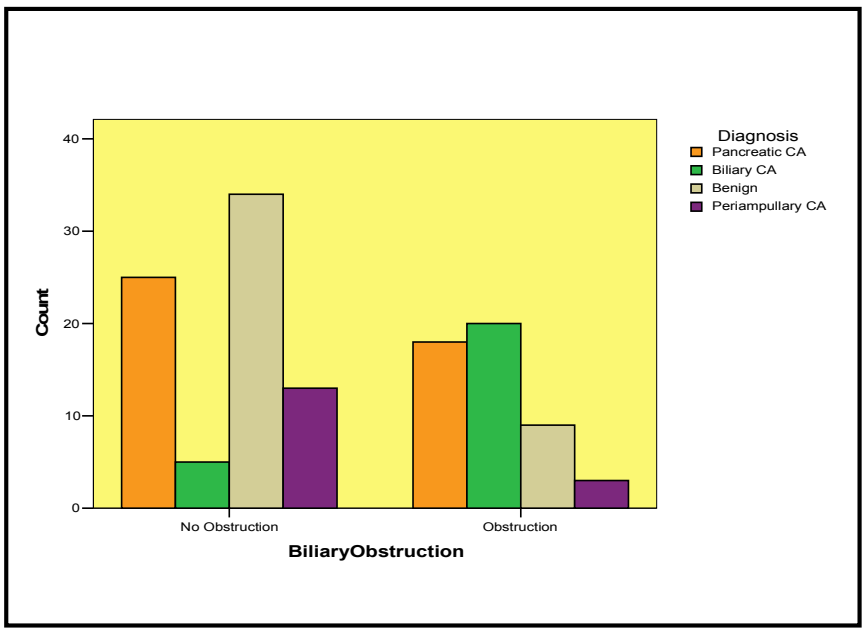

Figure 5.8: Distribution of patient depending on presence of biliary obstruction (serum bilirubin level >35 µmol/L)

As appears from the bar chart above (Figure 5.8), there seems to be a notable difference among the groups in the degree of biliary obstruction. A Chi square test was done on the cross table and it confirms that there are significant difference between the groups ($p<0.0001$).

Source of bile – common bile duct (CBD) or gallbladder (GB) – effect on biliary metal concentration

As explained earlier, bile sample for this study was collected from CBD or GB or from both sources whenever possible. It was reasonable to expect some change in concentration of the metals in the bile from gallbladder as gallbladder concentrates bile. However, no concrete evidence was available in the literature that there is significant difference in concentration of heavy metals excreted in bile sampled from the CBD and the GB. Unfortunately, due to rarity of the diseases investigated in the study, we had get the samples from whatever sources available and we could only get samples from both sources in patients undergoing resection for biliary obstruction. Thirty-three such patients were identified where we had sample from both the sources (66 samples). It was decided to use these samples to try and find a relation between the concentration in GB sample and the CBD sample of each metal. We decide to exclude the samples, which had evidence of biliary obstruction in order to avoid the possible influence of obstructed biliary system. 5 sets of samples were excluded due to inadequate volume or biliary obstruction and analysis was done using 28 set of samples. 4 metals were analyzed – zinc, copper cadmium and lead. Standard Pearson correlation analysis was done.

Zinc

Descriptive Statistics

	Mean	Std. Deviation	N
ZincCBD	.96068	.894167	28
ZincGB	1.39196	1.796878	28

Correlations

		ZincCBD	ZincGB
ZincCBD	Pearson Correlation	1	.532
	N	28	28
ZincGB	Pearson Correlation	.532	1
	N	28	28

Table 5.6: Zinc level from samples taken from gallbladder (GB) and common bile duct (CBD) – correlation statistics

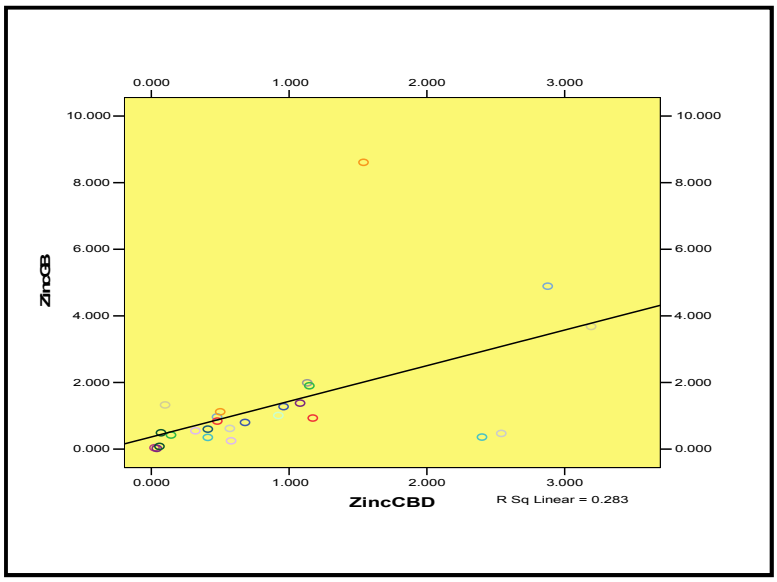

Figure 5.9: Graphical representation of correlation between zinc level of samples from gallbladder (GB) and common bile duct (CBD)

Pearson correlation coefficient was 0.532 for zinc obtained from CBD versus the samples from CBD (Table 5.6). The closer the value is towards 1.00, stronger is the correlation. The above value signifies moderate correlation

between the levels of zinc obtained from CBD and GB (Figure 5.9). Similar analysis was done for all the other 3 metals.

Copper

Descriptive Statistics

	Mean	Std. Deviation	N
CopperCBD	.6559	.55542	27
CopperGB	.7647	.69333	27

Correlations

		CopperCBD	CopperGB
CopperCBD	Pearson Correlation	1	.862
	N	27	27
CopperGB	Pearson Correlation	.862	1
	N	27	27

Table 5.7: Copper level from samples taken from gallbladder (GB) and common bile duct (CBD) – correlation statistics

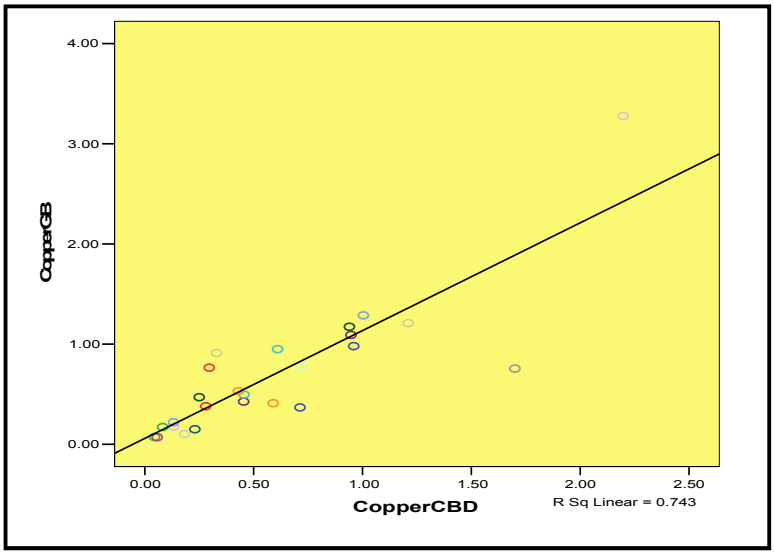

Figure 5.10: Graphical representation of correlation between copper level of samples from gallbladder (GB) and common bile duct (CBD):

The Pearson correlation coefficient for copper is 0.862 suggesting a strong correlation between the 2 values (Table 5.7, Figure 5.10).

Cadmium

Descriptive Statistics

	Mean	Std. Deviation	N
CadmiumCBD	.8151	.95143	24
CadmiumGB	.9680	1.18159	21

Correlations

		CadmiumCBD	CadmiumGB
CadmiumCBD	Pearson Correlation	1	.947
	N	24	21
CadmiumGB	Pearson Correlation	.947	1
	N	21	21

Table 5.8: Cadmium level from samples taken from gallbladder (GB) and common bile duct (CBD) – correlation statistics

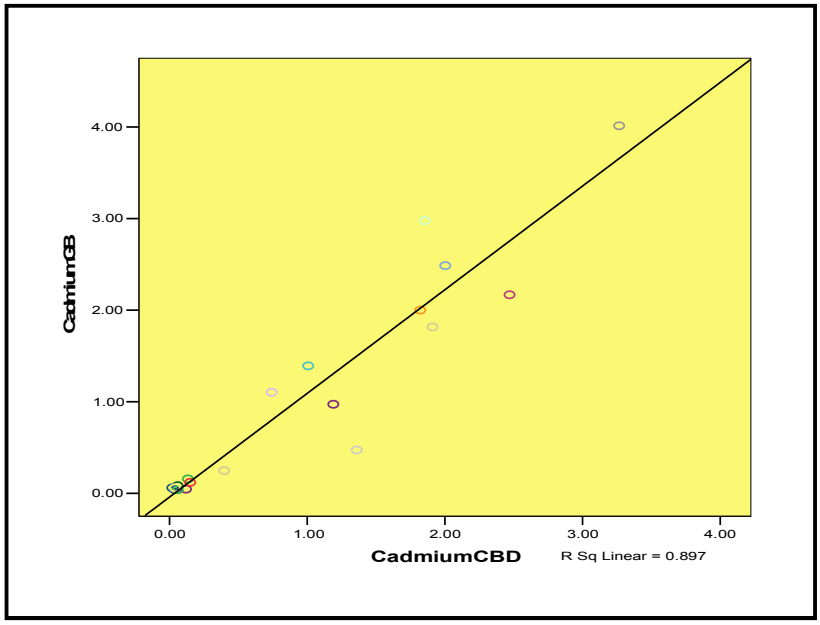

Figure 5.11: Graphical representation of correlation between cadmium level of samples from gallbladder (GB) and common bile duct (CBD)

The Pearson correlation coefficient for cadmium is 0.947 signifying a strong correlation between CBD and GB samples (Table 5.8, Figure 5.11).

Lead

Descriptive Statistics

	Mean	Std. Deviation	N
LeadCBD	.2266	.17862	14
LeadGB	.2571	.22615	15

Correlations

		LeadCBD	LeadGB
LeadCBD	Pearson Correlation	1	.675
	N	14	12
LeadGB	Pearson Correlation	.675	1
	N	12	15

Table 5.9: Lead level from samples taken from gallbladder (GB) and common bile duct (CBD) – correlation statistics

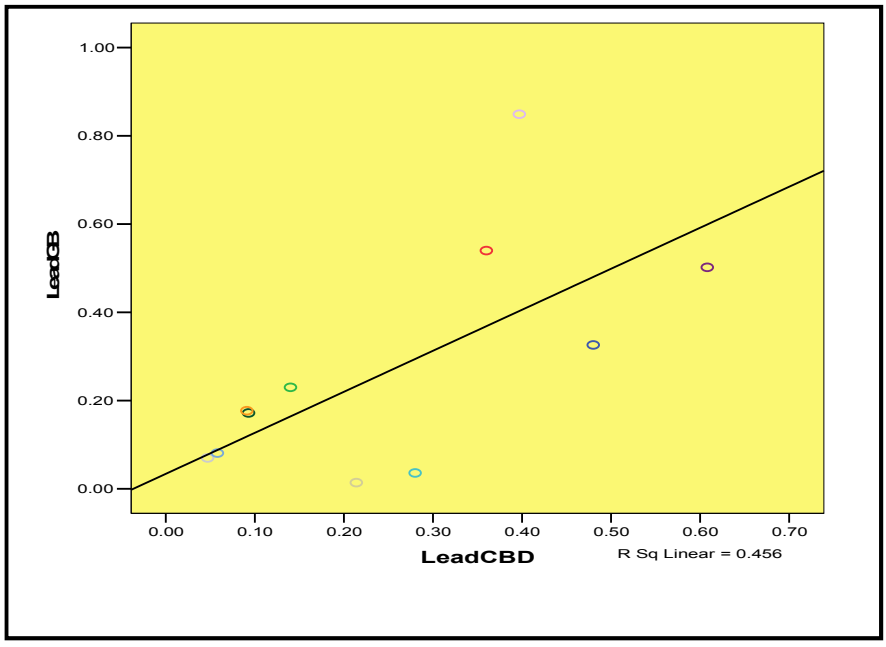

Figure 5.12: Graphical representation of correlation between lead level of samples from gallbladder (GB) and common bile duct (CBD)

The Pearson correlation coefficient for lead is 0.675, which suggest a fairly strong correlation (Table 5.9, Figure 5.12).

The above correlation study suggests that there is a correlation between the concentrations of the metals sampled from GB or CBD. However this correlation is variable and copper and cadmium samples seem to have the strongest correlation between the 2 sources. It is, however, not possible to comment if there is any statistically significant difference between the correlation of each metal.

We can assume from the above study that all the four metals under consideration, i.e. zinc, copper, cadmium and lead are excreted in bile in variable quantities. Although the bile is concentrated in the gallbladder compared to the bile in the common bile duct, no studies were found in literature in either human or animal model that has demonstrated that heavy metals are present at a higher concentration in bile obtained from gallbladder compared to the bile from common bile duct. The above analysis of correlation between the concentration of each metal sampled from GB or CBD suggests that there is probably no significant difference between the two sources. It is, therefore, proposed that for the final analysis of difference in heavy metal concentration between different study groups, we will perform the comparison taking one representative sample (from GB or CBD) from each patient in the group of 33 patients where we had sample from both sources (CBD or GB). The sample of bile taken from CBD is likely to be the more accurate representation of biliary excretion of heavy metal as this is the sample of bile directly secreted from liver without being stored or concentrated in the gallbladder. Comparison between different study groups

was, therefore, done using the sample from CBD in those subjects where we had sample from both sources. Each subject had one unique sample for comparison and final analysis.

Obstructed biliary system – effect on biliary metal concentration

Continuing the above discussion on possible effect of source of bile on the metal concentration, we also felt that the presence or absence of biliary obstruction may effect the heavy metal concentration. Patients with biliary obstruction are those patients with obstruction in flow of bile in the common bile duct (CBD). This is clinically manifested by jaundice and the obstruction can be caused buy the tumour itself in patients with malignant disease or by benign strictures or stones in patients with benign disease. The incidence of biliary obstruction is higher in the malignancy than in benign disease in general and the same is seen in our series (Figure 5.13). Therefore, when

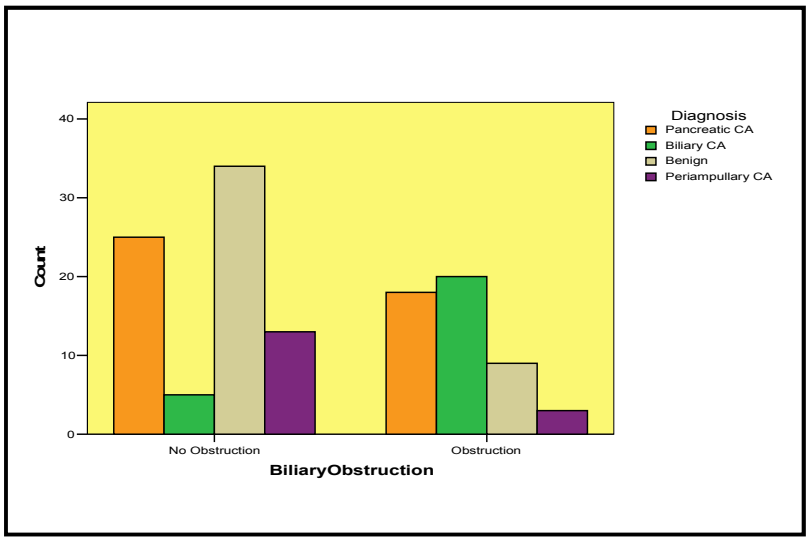

Figure 5.13: Distribution of patient depending on presence of biliary obstruction (serum bilirubin level >35 µmol/L)

we compare the heavy metal levels between the benign and malignant groups, the malignant group will have more patients with biliary obstruction

and it seems logical to explore the possible influence of biliary obstruction on the concentration of each heavy metal in our study.

The strategy for this analysis was similar to the one used for looking at influence of source of bile (CBD or GB) in the previous section, i.e., Pearson correlation analysis. The degree of biliary obstruction is represented by the bilirubin level and that was correlated with the concentration of each heavy metal in the study. A bilirubin level higher than 35 μmol/lit was selected as a sample with biliary obstruction in order better demonstrate the difference between obstructed and non-obstructed samples. This level of bilirubin is usually chosen in most studies to indicate biliary obstruction and is also used to select patients for clinical trials involving chemotherapeutic drugs. Each metal has been analyzed separately as in the previous section.

Zinc

The mean Level of zinc in all the samples was correlated with the bilirubin level of blood at the time of collection of the sample. As discussed the previous section, Pearson correlation coefficient is 1.00 when there is complete positive correlation between the 2 values being assessed (Table 5.10).

		Bilirubin Level	Zinc
Bilirubin Level	Pearson Correlation	1.000	-.094
	N	127	127
Zinc	Pearson Correlation	-.094	1.000
	N	127	127

Table 5.10: Correlation between bilirubin level and zinc concentration

Similarly, a value of -1.00 signifies a complete negative correlation. Pearson correlation coefficient for zinc in our experiment -0.094, signifying a rather weak negative correlation. The same fact is demonstrated in Figure 5.14, showing no linear relation between the mean level of zinc and bilirubin level.

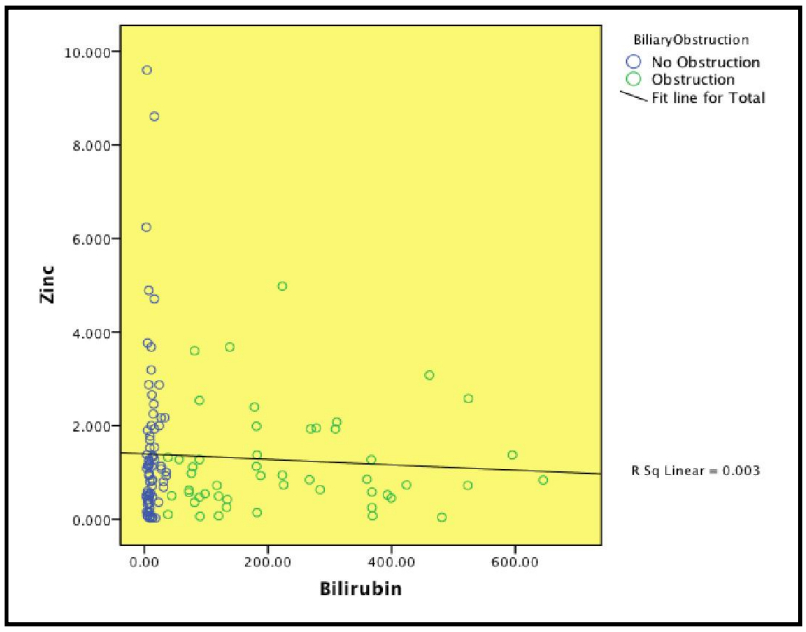

Figure 5.14: Graphical representation of correlation between bilirubin level and zinc concentration

Copper

Pearson correlation coefficient for copper in our study was -0.185, suggesting a weak negative correlation between copper levels and billirubin levels (Table 5.11). This is again shown in the diagram below (Figure 5.15).

		Bilirubin Level	Copper
Bilirubin Level	Pearson Correlation	1.000	-.185
	N	127	122
Copper	Pearson Correlation	-.185	1.000
	N	122	127

Table 5.11: Correlation between bilirubin level and copper concentration

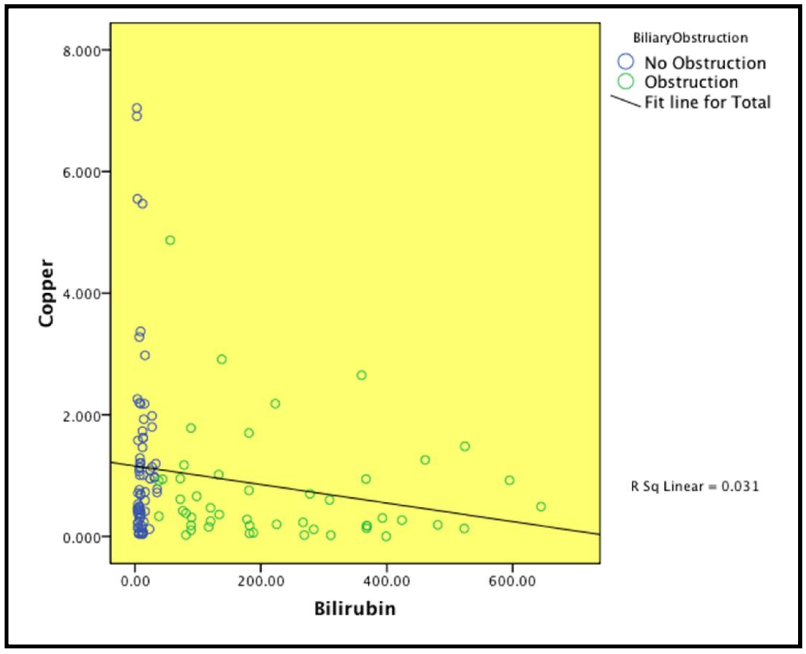

Figure 5.15: Graphical representation of correlation between bilirubin level and copper concentration

Cadmium

Like zinc and copper, cadmium also has a very weak, but this time positive correlation with the bilirubin level of the patient (Pearson correlation coefficient of 0.079) (Table 5.12). The scatter plot also shows widely distributed values with no linear relationship (Figure 5.16).

		Bilirubin	Cadmium
Bilirubin	Pearson Correlation	1.000	.079
	N	127	110
Cadmium	Pearson Correlation	.079	1.000
	N	110	110

Table 5.12: Correlation between bilirubin level and cadmium concentration

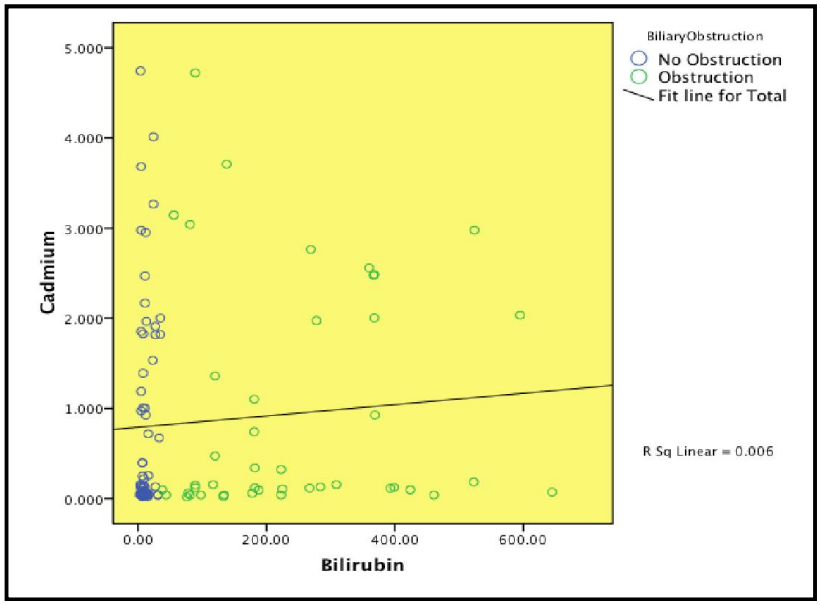

Figure 5.16: Graphical representation of correlation between bilirubin level and cadmium concentration

Lead

Pearson correlation coefficient for lead is 0.282. This follows the same trend as other metals with having a weak correlation with bilirubin level (Table 5.13). It is interesting to note that this value is closest to 1.0 amongst all the four metals and therefore lead has the strongest correlation with the bilirubin level in our study. This finding is demonstrated in the scatter plot where the values are more closely distributed around the line of best fit (Figure 5.17). Even then

		Bilirubin Level	Lead
Bilirubin Level	Pearson Correlation	1.000	.282
	N	127.000	74
Lead	Pearson Correlation	.282	1.000
	N	74	74.000

Table 5.13: Correlation between bilirubin level and lead concentration

the distribution is not linear and the correlation is weak. Any values above 0.5 are taken as strong correlation between 2 values in statistical terms.

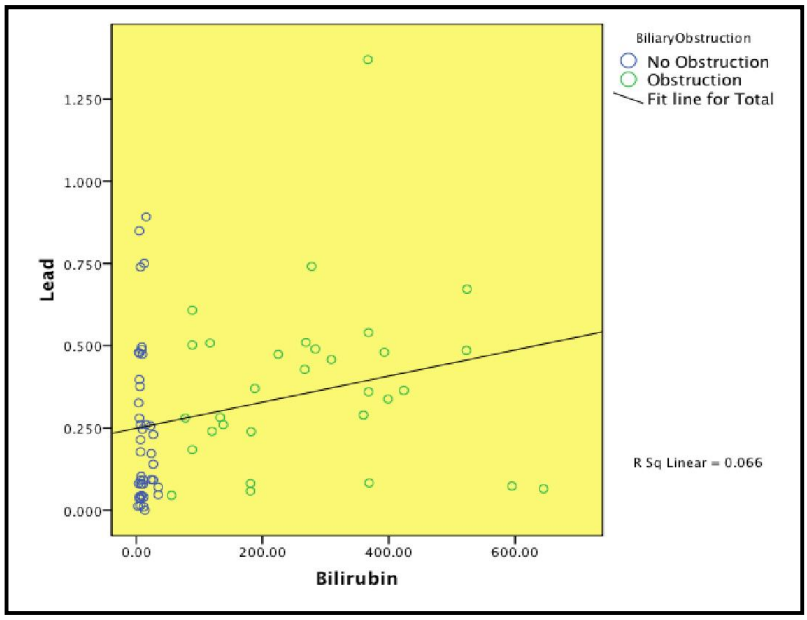

Figure 5.17: Graphical representation of correlation between bilirubin level and lead concentration

This lack of correlation between the mean level of heavy metals and the bilirubin in our study meant that the levels of heavy metals in the bile did not depend or get influenced by biliary obstruction, at least in our study population. It will not be reasonable to conclude from this analysis that such influence does not exist, but this analysis will allow us to ignore possible influence of biliary obstruction in our study when we compare the heavy metals levels between different study groups with benign and malignant diseases.

Comparison between groups for each metal – the final analysis

This analysis was done taking a single sample (CBD) from the group of 33 that had 2 samples (i.e using single primary sample from each patient). This was done to exclude 2 samples from the same patient, which would obviously skew the result. The CBD sample was considered the most representative sample as it is directly being excreted from the liver and not

being concentrated in the gallbladder. However, we have demonstrated the previous section that there is strong correlation between the metal levels from either sources in our study and therefore a representative sample can be taken from either sources for the final analysis.

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Zinc	Pancreatic CA	28	1.10357	.759031	.143443	.80925	1.39789	.059	3.192
	Biliary CA	22	1.17386	1.002924	.213824	.72919	1.61854	.040	3.769
	Benign	38	1.65937	1.971297	.319787	1.01142	2.30732	.023	9.600
	Periampullary CA	10	.80880	.777259	.245791	.25278	1.36482	.040	2.660
	Total	98	1.30479	1.416568	.143095	1.02078	1.58879	.023	9.600
Copper	Pancreatic CA	26	.62265	.921704	.180761	.25037	.99494	.048	4.871
	Biliary CA	22	.69223	.703798	.150050	.38018	1.00427	.000	2.650
	Benign	36	1.73267	1.880851	.313475	1.09628	2.36906	.039	7.044
	Periampullary CA	10	.79580	.618722	.195657	.35319	1.23841	.034	1.730
	Total	94	1.08247	1.405788	.144996	.79453	1.37040	.000	7.044
Cadmium	Pancreatic CA	25	.84864	1.063557	.212711	.40963	1.28765	.020	3.266
	Biliary CA	20	1.41875	1.448307	.323851	.74092	2.09658	.026	4.722
	Benign	34	.41497	1.002480	.171924	.06519	.76475	.020	4.743
	Periampullary CA	9	.78511	1.024381	.341460	-.00230	1.57252	.040	2.950
	Total	88	.80416	1.180871	.125881	.55396	1.05436	.020	4.743
Lead	Pancreatic CA	17	.29753	.194254	.047114	.19765	.39741	.041	.608
	Biliary CA	18	.45828	.301283	.071013	.30845	.60810	.065	1.370
	Benign	20	.21865	.255779	.057194	.09894	.33836	.000	.892
	Periampullary CA	4	.12550	.106221	.053111	-.04352	.29452	.040	.280
	Total	59	.30817	.266685	.034719	.23867	.37767	.000	1.370

Table 5.14: Raw data of each metal concentration among different groups of patients

The date used for analysis is shown in Table 5.14. A one-way ANOVA was performed to look for difference between groups. The advantage of using this test is that it is very useful in looking at differences in mean between more than 2 groups and can be used as a preliminary test to assess if there is any significant difference between the groups. At the end of the final study we had 4 distinct groups to look at and compare i.e. pancreatic cancer, biliary cancer, periampullary cancer and the benign control group. It was felt that ANOVA would be the most appropriate initial test to look at difference between the groups (Table 5.15).

		Sum of Squares	df	Mean Square	F	Sig.
Zinc	Between Groups	8.748	3	2.916	1.475	.226
	Within Groups	185.898	94	1.978		
	Total	194.647	97			
Copper	Between Groups	24.889	3	8.296	4.699	.004
	Within Groups	158.902	90	1.766		
	Total	183.790	93			
Cadmium	Between Groups	12.757	3	4.252	3.290	.025
	Within Groups	108.561	84	1.292		
	Total	121.318	87			
Lead	Between Groups	.701	3	.234	3.755	.016
	Within Groups	3.424	55	.062		
	Total	4.125	58			

Table 5.15: Results of ANOVA to look at the differences between groups of patients

The results of ANOVA suggested significant differences between groups for copper (p=0.004), cadmium (p=0.025) and lead (p=0.016). Form the ANOVA test itself it is impossible to comment whether any particular group is contributing to the significant difference among the groups. However, it helps us with the information that there is significant difference in the mean level of the 3 above metals between the groups and therefore it is justified to pursue this further by looking at each individual metal under study.

Another strategy was to look at the mean level of each metal in each group of the patient and that will give us and idea about possible influences by each group to the above significant difference on the ANOVA. The means plot for each metal shown below suggests that these significant differences are probably contributed by high level of copper in the benign group and high levels of cadmium and lead in the biliary cancer group (
Figure 5.18).

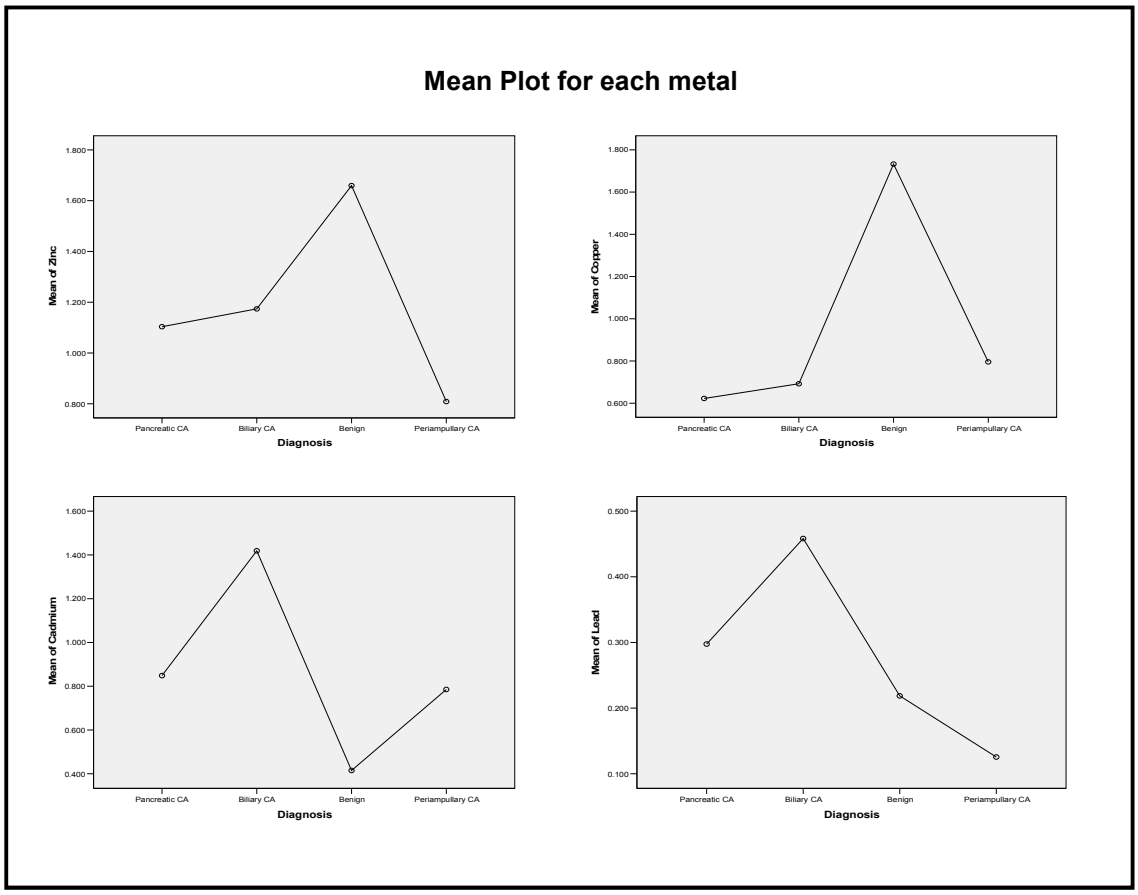

Figure 5.18: Means plot of zinc, copper, cadmium and lead concentration in each group of patients

On the basis of the above analysis, we planned to compare the mean or median concentration of each metal between the individual cancer groups versus the benign group to look for significant differences using non-parametric test (Mann-Whitney U test). As discussed in the pilot study, the median has been the preferred value in the analysis as the results are not normally distributed. The results for each metal are shown below.

Zinc

Box plot of zinc level shown in the figure suggests very similar concentrations in each group. There was no significant difference in the level of zinc between the groups when each of the cancer groups (pancreatic cancer, biliary cancer and periampullary cancer) was compared with the benign group by non-parametric test (Figure 5.19).

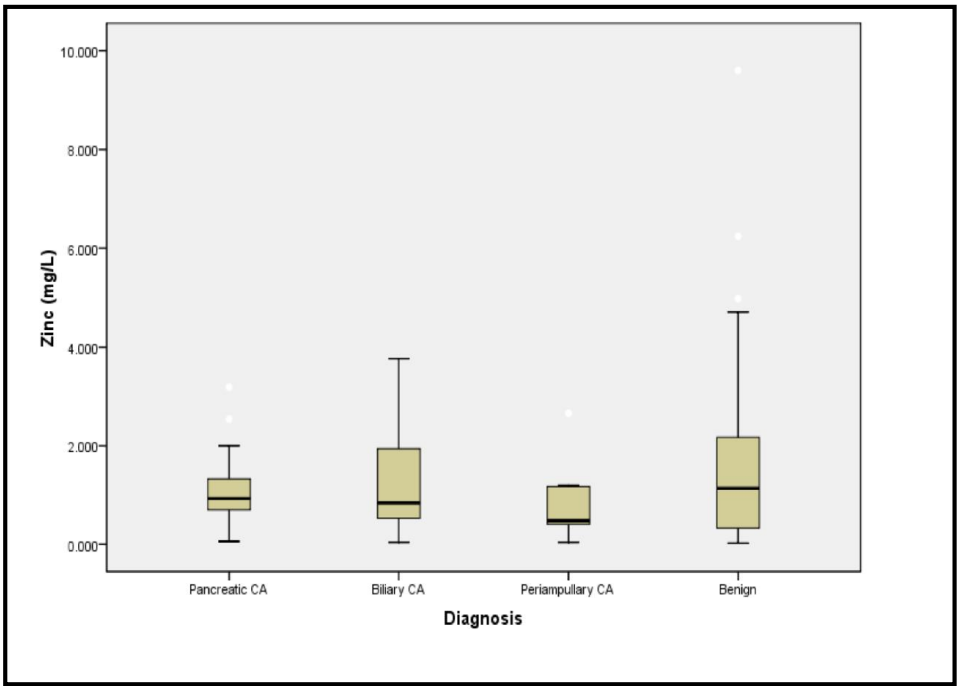

Figure 5.19: Box plot comparing median concentration of zinc between different groups of patients

Copper

Copper levels were compared in a similar fashion. The median level of copper was higher in the benign group. The median levels of copper were compared between the groups as with zinc. There was statistically significant difference in the levels of copper when comparing pancreatic cancer versus benign group (p=0.006) and biliary cancer versus benign (p=0.016). There

was no significant difference when periampullary cancer was compared against the benign group (Figure 5.20).

Cadmium

From the bar chart it appears that the median level of cadmium is higher in the biliary cancer group. The difference is statistically significant when compared to benign group (p=0.007). There is significant difference in cadmium levels when pancreatic cancer was compared with the benign

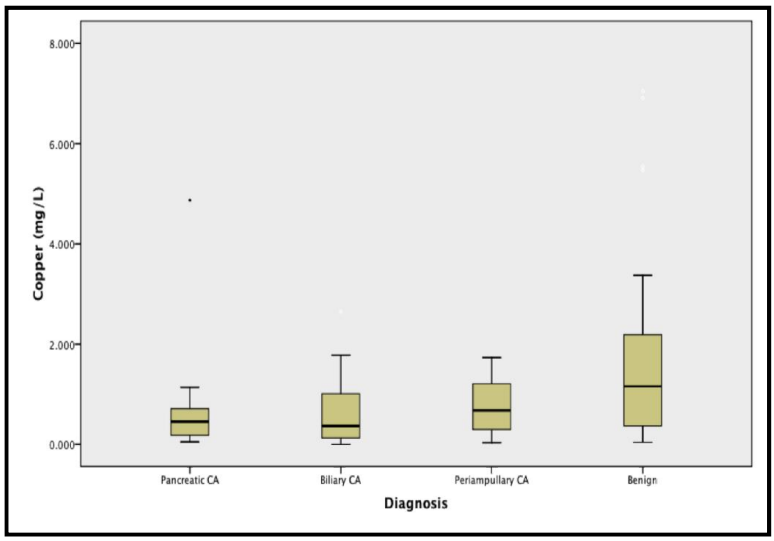

Figure 5.20: Box plot comparing median concentration of copper between different groups of patients

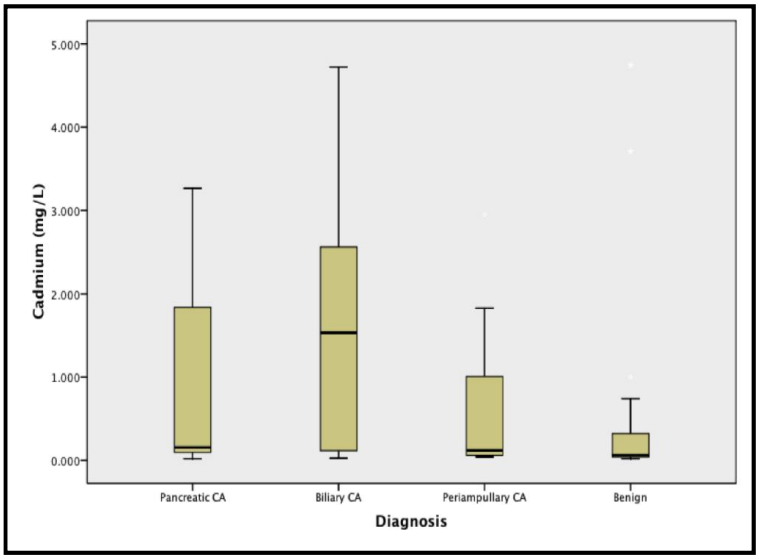

Figure 5.21: Box plot comparing median concentration of cadmium between different groups of patients

group (p=0.03). There was no difference when the periampullary cancer group was compared with the benign group (Figure 5.21).

Lead

From the figure below (Figure 5.22), the mean level of lead in biliary cancer group appears higher and when compared with the benign group there is a significant difference (p=0.005). There was no difference when pancreatic cancer and periampullary cancer were compared to the benign group.

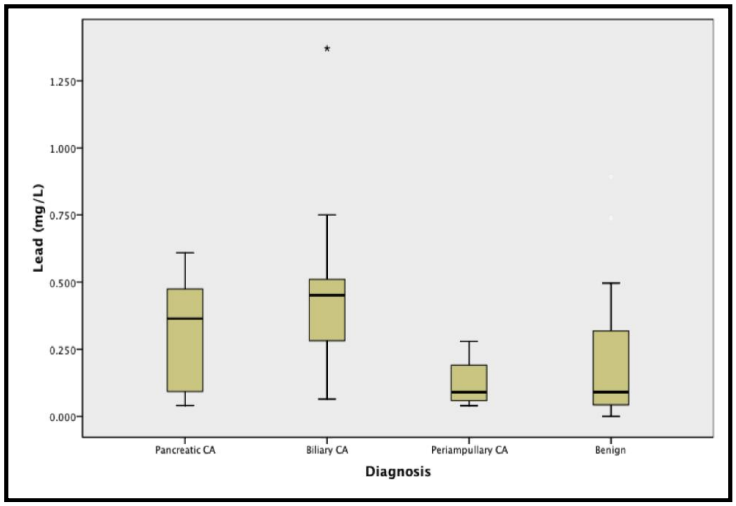

Figure 5.22: Box plot comparing median concentration of lead between different groups of patients

In summary, both cadmium and lead were present at a significantly higher concentration in the biliary cancer group compared to the benign control group. Cadmium was also present at a significantly higher level in pancreatic cancer group compared to the control. There are numerous reports of cadmium and its association with pancreatic cancer. The possible association of both cadmium and lead will be explored and discussed in the next section. There are also reports of copper and its association with biliary cancer, although that is not supported by this study.

Chapter 6

Discussion

6. Discussion

Secretory and excretory functions of bile

Bile is a detergent rich fluid secreted by the liver into the intestinal tract in almost all vertebrates. The gallbladder, a reservoir for bile in the form of a pouch from the biliary tree in between the liver and the intestine, is present in most vertebrates and some birds. Bile is not only stored in the gallbladder, but it is also concentrated and intermittently discharged into the biliary system during digestion. Along with saliva, gastric and pancreatic juice, bile helps in assimilation of food. As far as digestion of food is concerned, the most important constituent of bile is the bile salts. Bile salts are water-soluble end products of cholesterol metabolism formed by conjugating a bile acid to taurine and glycine. The anions of such conjugated bile salts are impermeable to cell membrane and have the ability to dissolve biliary phophatidylcholine, as well as breakdown products generated by pancreatic lipases. The final product of such lipolysis is mostly fatty acid. Long chain fatty acids are poorly soluble in the pH of the small intestine because of formation of acid soaps that have very low solubility at the slightly acidic media of jejunum. However, the partly ionized fatty acids readily form mixed micelles with bile salts. Solubilisation with micelle formation increases the amount of fatty acid present in the aqueous phase by a thousand fold. Because of their size, micelles diffuse more slowly than single molecule, but the increased amount of fatty acid present in this form increases the overall diffusion of long chain fatty acids. Fatty acids that are water-soluble do not require this mechanism for absorption.

As an excretory fluid, bile contains substances that cannot be eliminated efficiently in urine because they are either insoluble or protein bound. These include bile acids (as end products of cholesterol metabolism), bilirubin (end product of haem metabolism), and heavy metals such as iron, copper etc. The metals are usually derived from synthesis or absorption in excess of the needs of the body.

Physiology of bile secretion

Bile secretion starts in the canaliculus of hepatocytes ('canalicular bile'; about 75% of daily bile production) and is modified along the bile ductules and ducts ('ductular bile'; 25% of biliary secretion). Canalicular bile is formed by osmotic filtration of water and electrolytes in response to osmotic gradients

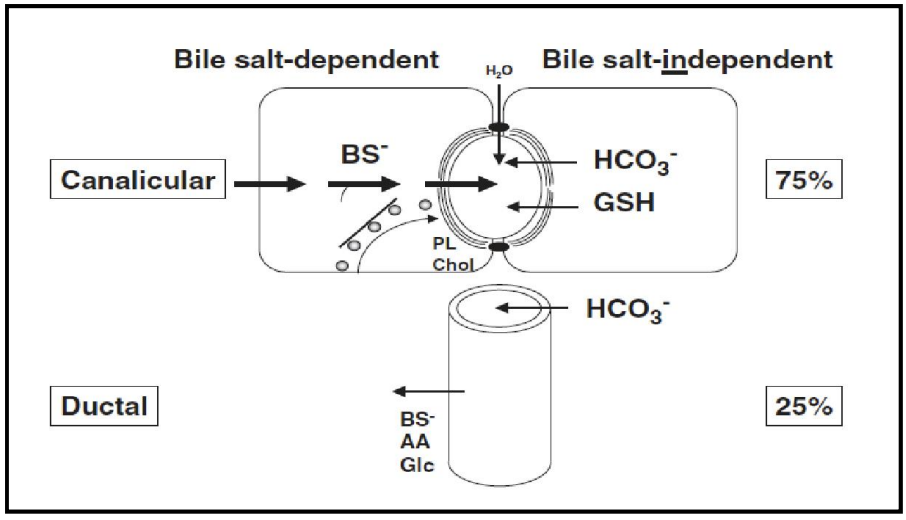

Figure 6.1: Mechanisms of bile formation. Bile formation begins at the canalicular level ('canalicular bile'; 75% of daily bile production), followed by modifications along the bile ducts ('ductal bile'; 25%)(Ferenci et al., 2002)

created by the active transport systems for bile salts and other organic anions, located at basolateral and canalicular membranes. The main determinant of canalicular bile formation is excretion of bile salts into the

canaliculus ('bile salt dependent' bile flow). Canalicular excretion of reduced glutathione (GSH) and bicarbonate (HCO_3^-) constitute the main component of the 'bile salt independent' fraction of the bile flow. Normal secretion of bile depends on

1. Active transport system across the membrane generating the osmotic gradient for bile flow.(Muller and Jansen, 1997, Muller and Jansen, 1998)
2. Intact cell morphology for movement of vesicles and bile canalicular contraction.
3. Cell contact and maintenance of cell polarity
4. Signal transduction mechanisms regulating various processes involved in bile formation (Figure 6.1) (Ferenci et al., 2002).

The active transport systems across cell membranes are of main interest in understanding heavy metal ion transport in the liver and their excretion in bile. The hepatocyte is polarized with basolateral (sinusoidal) and apical (canalicular) plasma membrane domains. Uptake of biliary constituents in the liver occurs at the basolateral membrane, which is in contact with sinusoidal blood via the space of Disse. A Na^+ - dependent Na^+/taurocholate co-transporter (NTCP) is the main transporter for bile salts (Hagenbuch and Meier, 1994, Hagenbuch et al., 1991). There is also an increasing number of Na^+-independent organic anion transporting protein (OATP) transporters not only for bile acids, but also for other organic anions like bilirubin and other cationic drugs. Na^+-dependent bile salt transport by NTCP is driven by a Na^+-K^+-ATPase which generates an inwardly directed Na^+ gradient (Kullak-Ublick, 1999).

The canalicular membrane contains both adenosine triphosphate (ATP)-dependent and ATP-independent transport systems. ATP-dependent transport systems (ATP binding cassette (ABC) proteins) transport constituents of bile against a concentration gradient. Most of these ABC proteins belong to the multi-drug resistance protein (MDR) or multi-drug resistance–associated protein (MRP) gene superfamily. There are several varieties of MDR/MRP with specific transport functions.

A conjugate export pump (MRP), functionally also known as the canalicular multispecific organic anion transporter (cMOAT), mediates canalicular excretion of a wide variety of organic anions, most of which are conjugated with glutathione, glucuronate and sulfate formed by conjugation in hepatocytes (Buchler et al., 1996). Other forms of MRP, such as MRP1 and MRP3 are also present in basolateral membrane and act as compensatory overflow system, especially under cholestatic condition with impaired canalicular MRP2 function (Konig et al., 1999, Kool et al., 1999). Mutations of genes coding for these transport proteins result in several hereditary cholestatic conditions. Although they are rare conditions, they provide interesting insight into biliary pathophysiology and help to understand the mechanism of various canalicular transport systems. Moreover, genetic transporter mutation and polymorphism may remain initially subclinical but may increase susceptibility to cholestatic challenges in adult life, for example during pregnancy or drug-induced cholestasis.

Understanding the mechanism of copper transport in the liver has been a major contribution to the knowledge of metal transport across biliary epithelium. Dietary copper intake far exceeds the normal daily requirement. Most of the ingested copper is taken up by the liver by a carrier mediated, energy independent mechanism (Figure 6.2) (Ferenci et al., 2002). In the hepatocytes, glutathione (GSH) has an affinity for copper and Cu^{+}-GSH complexes are formed. Cu^{+}-GSH works as an intracellular Cu^{+} transport and also acts as a donor to metallothionein and superoxide dismutase.

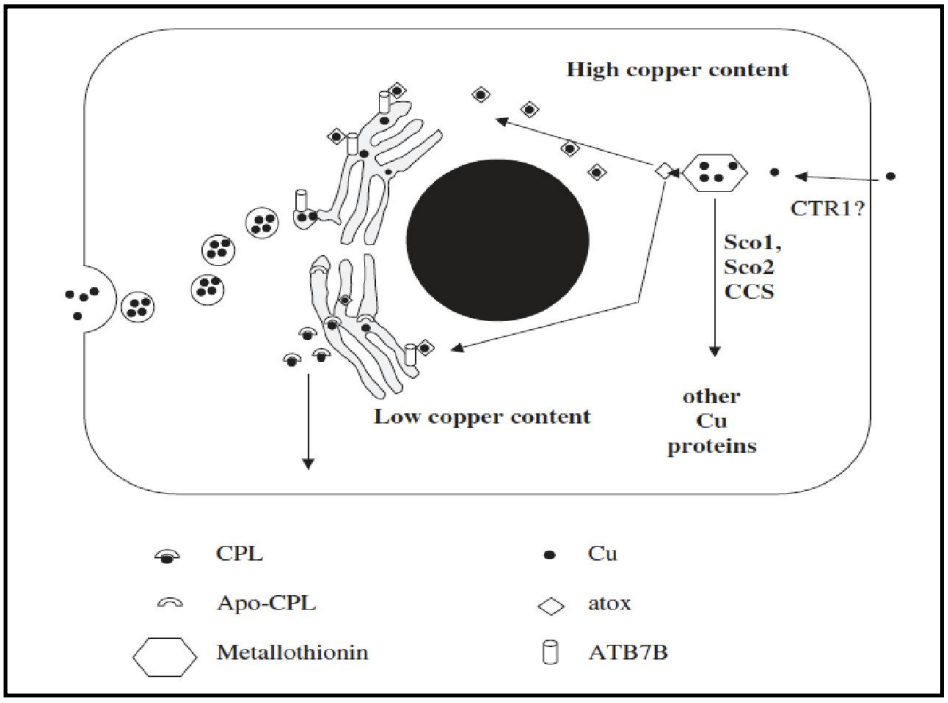

Figure 6.2: Copper transport in human liver. Copper taken up from blood (via CTR) is taken up by metallothionein, which is the storage protein for a variety of metal ions including zinc and copper (Ferenci et al., 2002)

Metallothionein is a low molecular weight, cytosolic, cystein-rich, metal binding protein. Copper stored in metallothionein can be donated to other proteins either by degradation in lysosomes, or by exchange through GSH-complex formation. Metallothionein plays a role in the homeostasis of essential metals such as zinc and copper, detoxification of toxic metals such

as cadmium and protection against oxidative stress. Metallothionein plays a minimal role in the gastrointestinal absorption of cadmium, but it plays important roles in cadmium retention in tissues and significantly decreases biliary excretion of cadmium. Cadmium bound to metallothionein is responsible for cadmium accumulation in tissues and the long biological half-life of cadmium in the body. Induction of metallothionein protects against acute cadmium induced death, as well as acute toxicity to the liver and lung. Intracellular metallothionein also plays important roles in ameliorating cadmium toxicity following prolonged exposures, particularly chronic cadmium induced nephrotoxicity, osteotoxicity, and toxicity to the lung, liver, and immune system. There is an association between human and rodent cadmium exposure and prostate cancer. Metallothionein expression in cadmium-induced tumors varies depending on the type and the stage of tumor development. For example, high levels of metallothionein are detected in cadmium-induced sarcomas at the injection site, whereas the sarcoma metastases are devoid of metallothionein (Klaassen et al., 1999). The use of metallothionein-transgenic and metallothionein-null mice has greatly helped define the role of metallothionein in cadmium toxicology, with the metallothionein-null mice being hypersensitive and metallothionein-transgenic mice resistant to cadmium toxicity (Liu et al., 2001). Thus, metallothionein is critical for protecting human health from cadmium toxicity. There are large individual variations in metallothionein expression, which might in turn predispose some people to cadmium toxicity (Klaassen et al., 2009). It is difficult to comment on the association of metallothionein expression and its relation to cadmium induced tumours or tumours that have

association with cadmium exposure. A study from India from an area of heavy metal pollution, reported that metallothionein expression was significantly higher in cases with carcinoma of the gallbladder (70.37%) as compared to chronic cholecystitis (25%) and normal gallbladders (0%). There was a trend suggestive of increasing metallothionein expression with increasing histological dedifferentiation of carcinoma of the gallbladder. The increased expression of metallothionein in cases of carcinoma of the gallbladder may represent an increased exposure to heavy metals, which are known carcinogens, and may have a role in gallbladder carcinogenesis. They speculated that metallothionein over-expression in carcinoma of the gallbladder may be relevant to the poor prognosis and chemo-resistance seen in these cases (Shukla et al., 1998a).

Heavy metal exposure and association with cancer

Although there is no clear definition of what a heavy metal is, density in most cases is taken as the most important factor. Heavy metals are commonly defined as having a specific density of greater than 5gm/cm^3. The main health hazards from heavy metal exposure are associated with lead, cadmium, mercury and arsenic. Heavy metals have been used in many different aspects of civilization for thousands of years. Lead has probably been used for nearly 5000 years with quite extensive use in ancient Rome. Famous artists like Claude Monet used cadmium pigments extensively in the1800s (Jarup, 2003). Although adverse effects of heavy metals have been known for a long time, environmental exposure to heavy metals continues and is even increasing. Since the middle of the 19th century, production of

heavy metals has increased steeply for more than 100 years with concomitant emissions to the environment (Figure 6.3) (Jarup, 2003).

At the end of the 20th century, heavy metal emission started to decrease in developed countries. In the UK, emission of heavy metals fell by over 50% between 1990 and 2000.

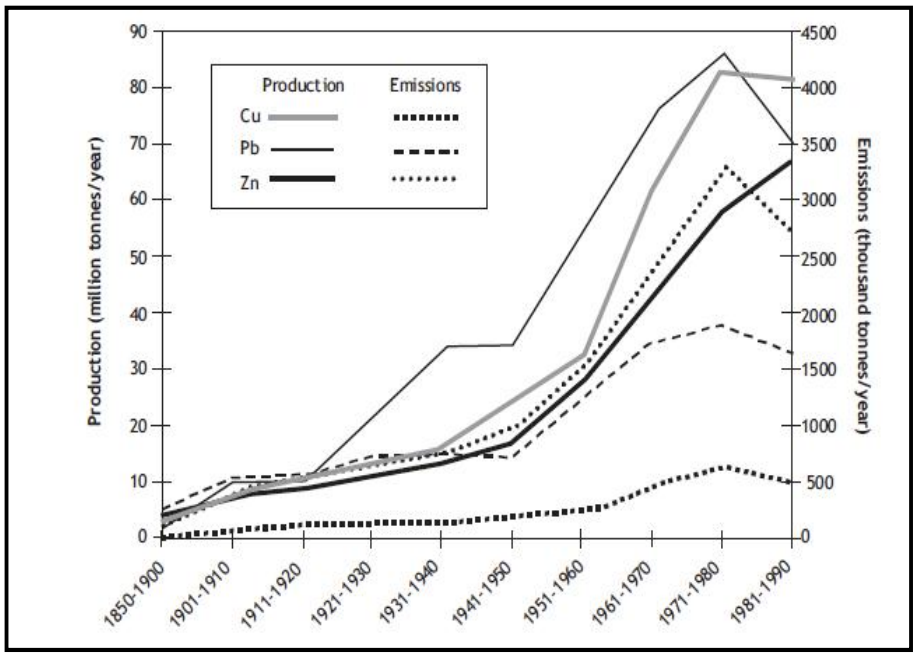

Figure 6.3: Global production and consumption of selected toxic metals, 1850–1990 (Jarup, 2003)

Heavy metal pollution can occur via different routes. The main sources of pollution are through air (during combustion in factories), surface water (effluent from storage and industry) and to soil and subsequently to groundwater and crops. Atmospheric emissions tend to be of greatest concern to human health, mainly because of widespread dispersion. The spatial distribution of cadmium and lead emission is shown below (Figure 6.4, Figure 6.5) (http://www.msceast.org/hms/emissions.html).

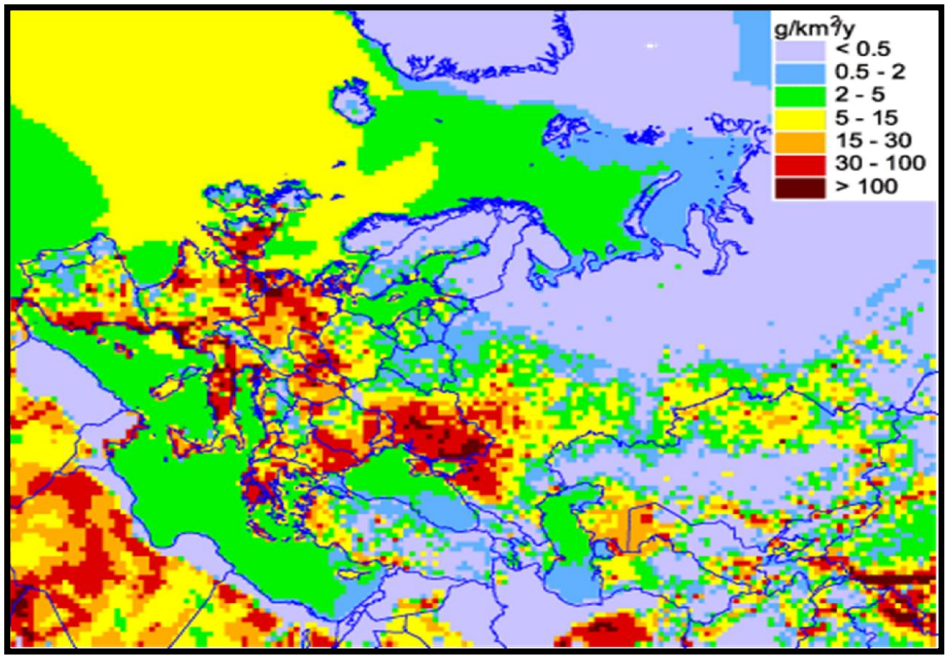

Figure 6.4: Spatial distribution of natural cadmium emissions in Europe in 2007, g/km2/y

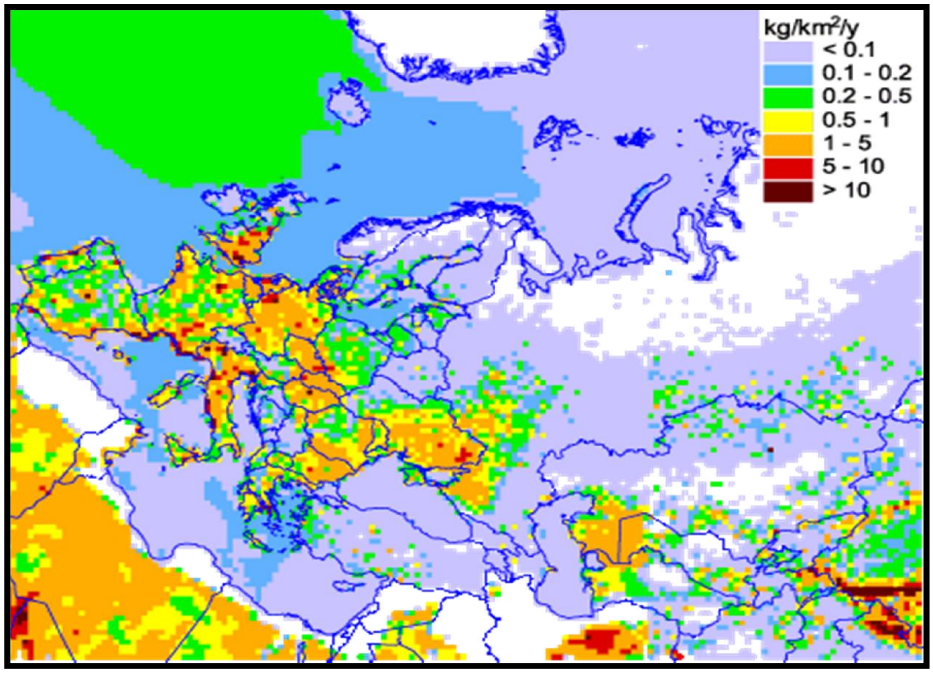

Figure 6.5: Spatial distribution of natural lead emissions in Europe in 2007, kg/km2/y

A population may be exposed to harmful chemical, physical and biological agents in air, food, water or soil. However, exposure does not result only from the presence of a harmful agent in the environment. There must be

contact between the agent and the outer boundary of the human body, such as the airways, the skin or the mouth. Exposure is often defined as a function of concentration and time: “an event that occurs when there is contact at a boundary between a human and the environment with a contaminant of a specific concentration for an interval of time” (Jarup, 2003).

In our study we are more interested in the possible carcinogenic effects of heavy metals. Non-carcinogenic adverse health effects of different heavy metals are excluded from discussion. Several metals and their compounds have been recognized as carcinogens: arsenic, beryllium, cadmium, chromium, cobalt, nickel and lead. The main route of exposure for most metals is inhalation and the main target organ is the lung. With the exception of hexavalent chromium, carcinogenic metals are weak mutagens, if at all, and their mechanisms of carcinogenicity are still far from clear. A general feature of arsenic, cadmium, cobalt and nickel is their property to enhance the mutagenicity and carcinogenicity of directly acting genotoxic agents. These properties can be interpreted in terms of the ability of these metals to inhibit the repair of damaged DNA. However, because carcinogenic metals cause tumor development in experimental animals even without other carcinogens, alternative mechanisms must be considered. There is evidence that carcinogenic metal compounds alter patterns of gene expression leading to stimulated cell proliferation, either by activation of early genes (proto-oncogenes) or by interference with genes down-regulating cell growth (Beyersmann, 2002).

Both scientific committees and regulatory agencies classify carcinogens. The table below summarizes classification of some metals and metalloids by the International Agency for Research on Cancer ((IARC)) and German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK commission). The IARC classifies the carcinogens according to carcinogenic hazard as carcinogenic to humans (Group 1), probably carcinogenic to humans (Group 2A), possibly carcinogenic to humans (Group 2B) or not classifiable as to its carcinogenicity to humans. The German MAK-commission also has classified several metals either as carcinogenic to humans (category 1), as considered to be carcinogenic to man based on long-term animal studies (category 2) or as giving concern to cause cancer, but evidence is not sufficient for classification in group 1 or 2 (category 3B) (Beyersmann and Hartwig, 2008) (Table 6.1).

Metals can be carcinogenic in the form of free ions, metal complexes, or particles of metal and poorly soluble compounds. The toxicity of metals depends on their physicochemical properties. Regarding pure metal ions, their oxidation state, charge and ionic radii are crucial. With metal complexes, their coordination number, the geometry and type of ligands are important for their toxic mechanism. Not only toxic metal cations, but also essential transition metal ions bind to biological ligands of opposite charges, such as amino acid side chains of proteins and phosphate groups of neocleotides and nucleic acids, and form complexes with oxygen, sulphur and nitrogen groups of proteins, nucleic acids and other biomolecules.

Substances	IARC carcinogen group	MAK carcinogen category
Antimony and its compounds	ø	2 (except SbH_3)
Antimony trioxide (Sb_2O_3)	2B	2
Antimony trisulfide (Sb_2S_3)	3	2
Arsenic and its compounds	1	1
Beryllium and its compounds	1	1
Cadmium and its compounds	1	1
Chromium metal	3	ø
Chromium(VI) compounds	1	2 (except $ZnCrO_4$: cat. 1)
Chromium(III) compounds	3	ø
Cobalt and its compounds	2B	2
Cobalt with tungsten carbide (hard metal)	2A	1
Gallium arsenide	1	ø
Indium phosphide	2A	2
Lead metal	ø	2
Lead compounds	2A	2
Mercury and its compounds	2B	3B
Nickel metal	2B	1
Nickel compounds	1	1
Rhodium	ø	3B
Selenium and its compounds	3	3B
Vanadium and its compounds	ø	2
Vanadium pentoxide (V_2O_5)	2B	2

Table 6.1: Classification of metals and/or their compounds as carcinogenic. Source: International Agency for Research on Cancer ((IARC)) Monographs, MAK (German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area). (ø, not classified)

example, Cd^{2+} ions have ionic radii very similar to those of Ca^{2+}. Although the preferred ligand of Ca^{2+} is oxygen, whereas Cd^{2+} prefers sulfur, Cd^{2+} also accepts oxygen and is able to substitute Ca^{2+} in protein binding sites. Cd^{2+} interferes with functions of numerous Ca^{2+}-transport and Ca^{2+}-dependent signalling proteins. In some cases substitution of Ca^{2+} by Cd^{2+} may even yield the normal protein function such as in the protein calmodulin where this substitution preserves 90% activity with cyclic phosphodiesterase (Cheung, 1984, Beyersmann and Hartwig, 2008). Cd^{2+} also have an analogous electron configuration with Zn^{2+} and can substitute Zn^{2+} in zinc enzymes and

alter the biochemical functions of such proteins. Similarly lead (Pb^{2+}) also interferes with many Ca^{2+}-regulated biochemical systems.

General mechanism of metal carcinogenicity

The toxicity of metals and their compounds largely depend on their bioavailability, i.e. the mechanism of uptake through cell membranes, intracellular distribution and binding to cellular macromolecules. The anionic compounds of chromium and vanadium smoothly penetrate into cells via the general anion channel of the plasma membrane. Hence, anionic chromium(VI) is readily taken up into cells but as soon as it is reduced to chromium(III) by intracellular reducing agents, the metal accumulates in the

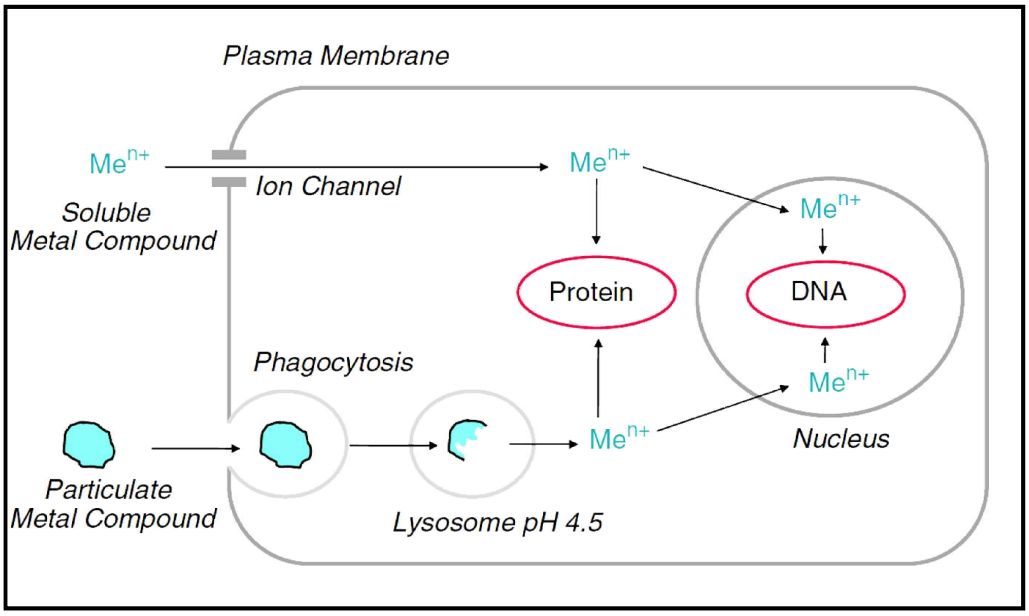

Figure 6.6: Cellular uptake, intracellular distribution and binding of soluble and particulate metal compounds (Beyersmann and Hartwig, 2008)

intracellular compartment (Cupo and Wetterhahn, 1985). Many toxic metals as divalent cations can penetrate the plasma membrane via cation cotransporters. In addition, soluble metal compounds may be taken up by phagocytosis leading to intracellular accumulation (Figure 6.6).

There appear to be three common pathways that contribute to the mechanism of carcinogenicity in majority of the metals, namely oxidative stress, DNA repair modulation and disturbances of signal transduction pathways.

Induction of oxidative stress

Oxidative stress is an interesting hypothesis to explain mutagenic and carcinogenic effects of metals. Ions of suspected carcinogenic metals like nickel and cobalt are capable of performing redox reactions in biological systems. They can induce formation of reactive oxygen and nitrogen species in vivo and vitro. Formation of hydroxyl radicals has been detected in experimental models and these radicals can cause oxidative damage to lipids, protein and DNA (Figure 6.7). Although ions of cadmium are not capable of inducing the same redox reaction in vivo, they can also generate oxidative stress through a different pathway. Cadmium can inhibit antioxidative enzymes in vivo and in vitro. Cadmium has been shown to inhibit catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase. Apart from generating DNA damaged directly, reactive oxygen species at low concentrations function as mitogenic signals and active redox sensitive transcription factors (Genestra, 2007). Therefore, oxidative stress can initiate tumour development by mutagenesis and also deregulate cell growth and promote tumour growth.

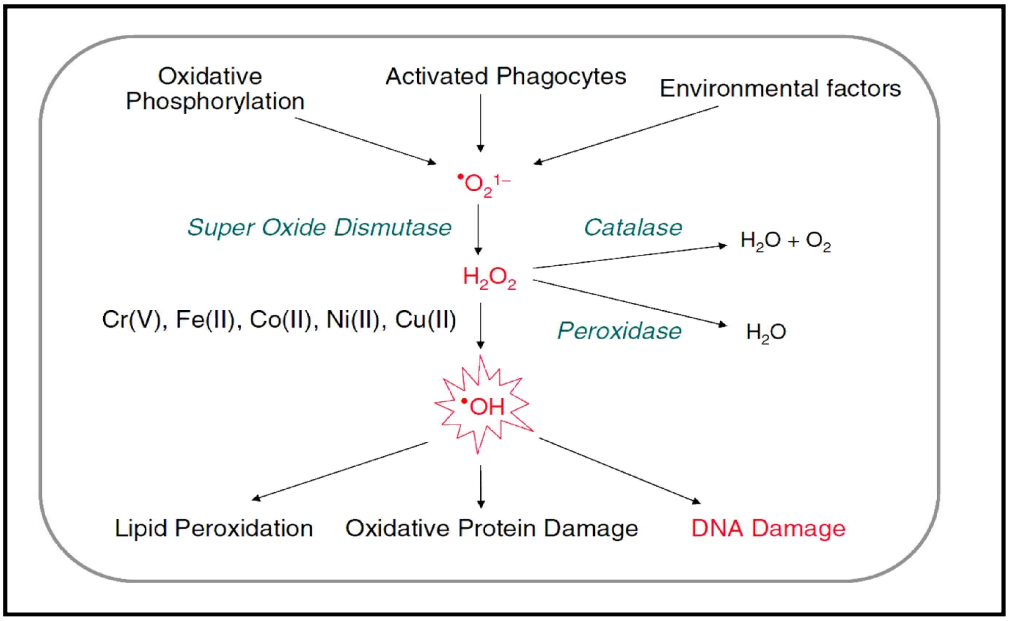

Figure 6.7: Metal ions and oxidative stress. Modified from (Beyersmann and Hartwig, 2008)

One argument against oxidative stress hypothesis of metal carcinogenicity is the discrepancy between the comparatively high, often cytotoxic doses of metal compounds that are required to evoke the formation of reactive oxygen species and the measurable increase in cellular structures and the often very low doses of metals that can induce tumours. It probably supports the possibility that the whole mechanism of carcinogenicity is multi factorial.

Interference with DNA repair

Carcinogenic metals are considered only weak mutagens in mammalian cells and often inactive in bacterial assays. Mutagenicity in bacterial assay is an indicator of reactivity of a substance with DNA, metals are thought to exert genotoxicity by indirect mechanism. They often show the mechanism of comutagenicity, i.e. they enhance the mutagenicity of other genotoxic agents. There are 4 main, partly overlapping DNA repair pathways in mammalian cells: base excision, mismatch, nucleotide excision and recombinational

repair. Either inherited or acquired defects of these pathways can initiate malignant growth. These pathways are possibly targeted by toxic metals and inhibition of repair and persistent DNA damage results in genomic instability, which may become more significant under conditions of cell proliferation (Beyersmann and Hartwig, 2008).

Deregulation of cell proliferation

Tumour development is characterized by deregulation of cell growth and differentiation. Carcinogenic metals alter cell growth by different mechanisms, either affecting the growth stimulating factors or inactivating growth control mechanisms. Some metal ions can induce expression of cellular proto-oncogenes and modify pattern of gene expression. Changes in gene regulation precede formation of tumours. Some metal carcinogens have been shown to inactivate tumour suppressor protein p53. Finally, metal ions can deregulate cell proliferation by inactivating apoptotic processes resulting in adaptation to the cytotoxicity of the metal (Beyersmann and Hartwig, 2008).

Cadmium and its role in pancreatobiliary malignancy

Human cadmium exposure is associated with cancers of the lung and kidney((IARC), 1993). Cadmium is a toxic metal occurring in the environment naturally and as a pollutant from industrial and agricultural sources. Smoking is one of the major source of cadmium inhalation and food and water are the main source in the non-smoking population. Cadmium is efficiently retained in the kidneys with a very long half life of 10 – 30 years, although cadmium is nephrotoxic and initially causes renal tubular damage. Cadmium occurs

naturally in ores together with zinc lead and copper. Cadmium compounds are used as stabilizers in PVC products, colour pigment, several alloys and most commonly, in-rechargeable nickel cadmium batteries. Cadmium is also present as pollutant in phosphate fertilizers. European Union (EU) cadmium usage has decreased considerably during the 1990s, mainly due to phase out of cadmium products other than Ni-Cd batteries and the implementation of more stringent EU environmental legislations. In spite of these changes in Europe, overall cadmium production, consumption and emissions to the environment worldwide has increased dramatically in the 20th century. Having a long half-life in the human body, the adverse effects of increased cadmium exposure will only be apparent at a later stage. Cadmium containing products are rarely recycled and frequently dumped together with household waste, thereby contaminating the environment (Jarup, 2003).

Cigarette smoking is a major source of cadmium exposure. The serum concentration of cadmium in smokers are on an average 4 – 5 times higher than those of non-smokers (Jarup et al., 1998). Food is the main source of cadmium in the general non-smoking population. Cadmium is present in most foodstuffs, but the concentration is highly variable. Gastrointestinal absorption of cadmium may be influenced by nutritional factors, such as the iron store (Flanagan et al., 1978). Serum cadmium levels generally reflect current exposure, but the cadmium concentration in the urine is influenced by the lifetime body burden and is proportional to the renal concentration of cadmium.

As part of our study, we are interested in the possible carcinogenic role of cadmium, especially its role in pancreatobiliary malignancy. As noted earlier, human cadmium exposure may be associated with cancers of the lung and kidney. In animals, cadmium induces carcinoma of lung after inhalation and cancers of the prostrate after ingestion of cadmium (Waalkes, 2003). Carcinogenicity of cadmium is due to multiple factors as explained earlier, i.e. indirect induction of oxidative stress, inhibition of DNA repair and deregulation of cell proliferation. In addition, cadmium has a unique mechanism of disruption of the cadherin mediated cell-cell adhesion system and cell-cell communication. Cadmium specifically displaces calcium from the protein E-cadherin (Prozialeck et al., 2003) and impair the cell-cell adhesion thereby deregulating cell proliferation.

There is very little literature about possible carcinogenic effect on pancreatobiliary malignancy. In recent years some work has been done to look at possible association between occupational or environmental exposure to cadmium and pancreatic cancer in areas of high incidence of this disease. There are several risk factors implicated for pancreatic cancer but they can be generally grouped in 4 categories:

1. Smoking
2. Chronic pancreatitis and genetic predisposition
3. Diabetes mellitus and micro and macronutrients
4. Occupational and environmental exposure to carcinogens

There is a lack of publications looking at the possible environmental exposure and pancreatic cancer. Ojajärvi et al. in a meta-analysis tried to

consolidate epidemiological data on pancreatic cancer and worksite exposure (Ojajarvi et al., 2000). Over a long period from 1969 to 1998, they looked at 92 publications covering 161 different population groups, looking at 23 agents or group of agents. Exposure to chlorinated hydrocarbon(CHC), nickel, chromium, polycyclic aromatic hydrocarbons and organochlorine insecticides were found to be associated with increased risk of pancreatic cancer. In this meta-analysis, cadmium compounds had very weak or no association with pancreatic cancer. The same group published in 2007 applying a hierarchical Bayesian meta-analysis model and identified CHC as the agent that is associated with increased risk of pancreatic cancer (Ojajarvi et al., 2007).

The strongest evidence of association between cadmium exposure and pancreatic cancer emerged from the East Nile Delta region of Egypt. This region exhibits a high incidence of early onset pancreatic cancer. The incidence of pancreatic cancer in this region for patients younger than 65 years is more than twice as high as those observed in Americans in the same age group and significantly higher than those seen in other parts of Egypt (Soliman et al., 2002). The reason for such a high incidence of early-onset pancreatic cancer in this region is unclear. There is no good evidence that the first 3 risk factors for pancreatic cancer noted above would preferentially affect the residents of this region relative to rest of Egypt. However, it is well documented that this region has one of the highest levels of environmental pollution in Egypt.

Kriegel at al. conducted a pilot study in this region of Egypt to assess serum cadmium level as marker of exposure in pancreatic cancer patients and compare it with non-cancer patients from the same region. Nile river water is seriously contaminated with heavy metals, pesticides and hydrocarbons due to discharge of untreated industrial waste. High concentration of heavy metals, including cadmium is pollutants in the water. Plants and fish that grow in this water are also contaminated by these metals, which in turn accumulate in humans that feed on these contaminated foods. The serum cadmium level of residents of this region is about 10 times that of residents from cadmium polluted areas in Cairo and about 32 times higher than reference levels for the healthy population in the United States (Kriegel et al., 2006). As discussed before, cadmium has a carcinogenic effect demonstrated both in vivo and in vitro and the 2 main risk factors of pancreatic cancer, namely smoking and age, are also associated with higher cadmium levels in the body. In this pilot study, serum cadmium levels and the occupation of farming were strong independent risk factors for pancreatic cancer.

Suspicion of a strong association between cadmium exposure and pancreatic cancer has been reported from Louisiana (Lemus et al., 1996). Industrial activity along the Mississippi River has led to an accumulation of contaminants downstream in southern Louisiana (Mielke et al., 2000). Louisiana residents are exposed to environmental cadmium by inhalation. Lemus et al have shown that 64 out of 315 samples (20.3%) of indoor and outdoor air from 53 households in Louisiana exceeded the U.S.

Environmental Protection Agency's permissible levels for cadmium (Lemus et al., 1996). Seafood and rice are popular food items in the local diet and both rice and fish harvested from cadmium-polluted areas contain high levels of cadmium. A case-control study from Louisiana showed significantly increased risk of pancreatic cancer associated with rice consumption among Cajuns, with a dose-response relationship (Falk et al., 1988, Falk et al., 1990). More recent studies based on the same population have shown an increased risk of pancreatic cancer with increased urinary cadmium concentration (Luckett et al., 2012).

Evidence is less convincing for an association of heavy metals and biliary cancers (gallbladder cancer and cholangiocarcinoma). The only evidence is available from an area of high incidence of gallbladder cancer and heavy industrial pollution in Northern India. In a case control study, Shukla et al demonstrated higher mean concentrations of cadmium, chromium and lead in the bile of patients with gallbladder cancer compared to a non-cancer group (Shukla et al., 1998b). A review article by Pandey et al. emphasized very little evidence on role of environmental pollutants in gallbladder cancer and highlighted the need for a robust epidemiological study (Pandey, 2006).

Report from the same region of India suggested an association between serum copper and zinc level and gallbladder cancer. In a study comparing patients with gallbladder cancer and benign biliary disease, the mean serum zinc levels were significantly lower in patients with carcinoma of the gallbladder than in patients with cholelithiasis and in healthy controls. The

mean serum copper levels were significantly higher in patients with carcinoma of the gallbladder as compared to patients with cholelithiasis and healthy controls. Biliary and tissue zinc levels were significantly lower in patients with carcinoma of the gallbladder than in patients with cholelithiasis. Biliary and tissue copper levels were higher in patients with carcinoma of the gallbladder than in patients with cholelithiasis (Gupta et al., 2005). Copper is an essential metal and plays the role of a co-factor for many biochemical reactions as a catalyst. Catalytic copper, because of its redox activity, can potentially play a central role in the formation of reactive oxygen species that bind rapidly to DNA causing DNA damage and thereby leading to carcinogenesis (Theophanides and Anastassopoulou, 2002). The present study, however, shows a higher concentration of copper in the bile of the malignant group and this is against the finding of Gupta et al. (2005) in the study discussed above.

In recent years prospective data collected from the Third National Health and Nutrition Examination Survey (NHANES III) in the Unite States has been used to investigate the relationship between cadmium exposure and cancer mortality and association of specific cancers with cadmium exposure in the general population (Adams et al, 2012). The cadmium exposure of the study population was assessed by measuring a spot urine sample collected from participants and assayed for cadmium and creatinine. Urinary cadmium concentration is a reliable marker of long term cadmium exposure (Lauwerys et al, 1994). Cadmium concentration was divided by creatinine to correct for variation in hydration and was recorded as creatinine-normalised urinary

cadmium (uCd, µg/g). The study observed association between uCd and cancer mortality in both men and women. This association with total mortality as well as cancer specific mortality was stronger in men than in women. This study also observed association between cadmium exposure and pancreatic cancer as reported in earlier studies (Schwartz et al, 2000) from occupational cohorts. Since cigarettes are a major source of cadmium among smokers and a cause of pancreatic cancer, the authors restricted the analysis to never-smokers and the association of uCd with pancreatic cancer remained. Therefore, the association of cadmium exposure to pancreatic cancer is possibly independent of smoking. Even low to moderate cadmium exposure, as measured in urine, was associated with mortality from overall, smoking-related, lung and pancreatic cancer in American-Indian adults who participated in the Strong Heart Study during 1989 to 1991 and followed up for 20 years (Garcia-Esquinas et al., 2014).

Cadmium exposure induces lung and pancreatic cancer in experimental rodent models (Huff et al., 2007, Waalkes, 2003). *In vitro,* chronic cadmium exposure to human pancreatic duct epithelial cells can result in malignant cell transformation with increased secretion of metalloproteinases, increased invasiveness and increased colony formation (Qu et al., 2012).

Environmental pollution in the North-East of England

Compared to areas of heavy industry and pollution along the rivers Nile, Mississippi and Ganges, river basins in the UK are areas of relatively low environmental pollution. However, one cannot ignore that several of these river basins, especially in the North of England, were areas of mining

activities and that in itself can lead to metal pollution downstream. Some of these basins are still areas of heavy industry, for example Teesside region in the North-East of England. In our study, although there was a significantly higher level of lead and cadmium in the cancer group, no significant regional variation was noted and there was no suggestion that areas of heavy industry like Teesside had a higher incidence of pancreatobiliary malignancy. However, this study was not adequately powered to look at this aspect.

Pless-Mulloli at al (Pless-Mulloli et al., 1998) assessed whether deprived populations living close to industry experience greater mortality from lung cancer than populations with comparable socioeconomic characteristics living further away in the North-East of England. Analysis was based on 2 areas; Teesside and Sunderland. Housing estates in Teesside were selected based on socioeconomic criteria and distinguished by proximity to steel and chemical industries; they were then grouped into 3 zones, i.e. near (A), intermediate (B) and further (C). The estates in Sunderland were grouped into a single zone (Figure 6.8).

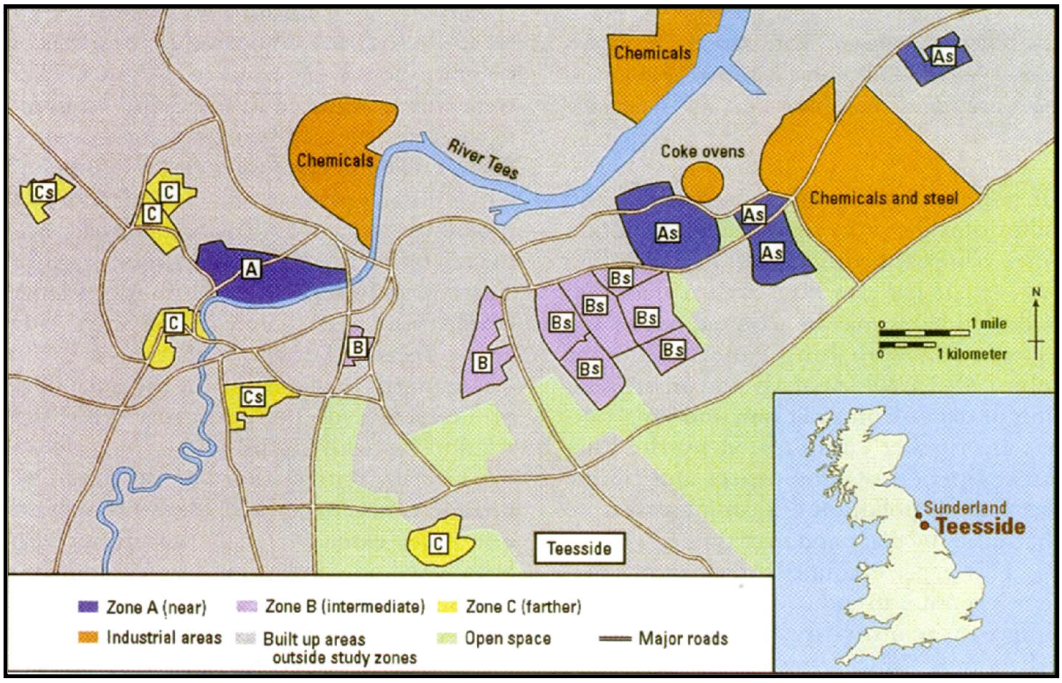

Figure 6.8: Estates selected for the analysis of mortality and survey data. Zone A, B and C indicate varying proximity to industry (Byrnes and Afdhal, 2002).

Mortality from lung cancer was well above national average in all zones. For men a weak gradient corresponding to the proximity to industry at younger age reversed at older age. In women between 0 to 64 yrs of age, a stronger gradient in lung cancer mortality corresponded with proximity to industry across zones A, B and C. Overall rates in Teesside were higher than Sunderland rates for women aged 0 - 64 yrs. The association between increased lung cancer mortality and proximity to industry in women under 75 years could not be explained by smoking, occupation, socio economic factors and occupation. Explanations for differences between men and women may include gender specific occupational factors and smoking patterns. The authors felt that the observed gradient in women points to a role for industrial air pollution.

The UK Environment Agency has recently published a report entitled Assessment of Metal Mining-Contaminated River Sediments in England and Wales (Science Report: SC030136/SR4, November 2008). The Environment Agency is the leading public body protecting and improving the environment in England and Wales and their work includes assessing and reducing the impact of industry on the environment.

Many river basins in England and Wales have been significantly contaminated with metals released from past mining operations. The Water Framework Directive (WFD) River Basin Characterisation exercise in 2005 estimated that 453 surface water bodies in seven of the eleven River Basin Districts were at risk of pollution by abandoned mines including coal mines. The peak period of metal mining was the mid to late 19th century, although pollution continued to be discharged from many sites. In the catchments, as much as 90% of the metals are associated with sediments rather than in aqueous form, and metal contaminants are mobilized and transported downstream and deposited by the river processes. This has created a collection of highly contaminated sediments, often a considerable distance downstream from the mines themselves. The report by the Environment Agency set out to use data from selected catchments to test the potential environmental impact of these contaminated sediments. It did not attempt to ascertain acceptable contaminant concentration in soil or water. Therefore, the report highlights the hazards associated with metal contaminated sediments and soil, but it did not evaluate the consequent risk. Over

thousands of years many tonnes of metals have been extracted from several major mining areas in the UK (Figure 6.9).

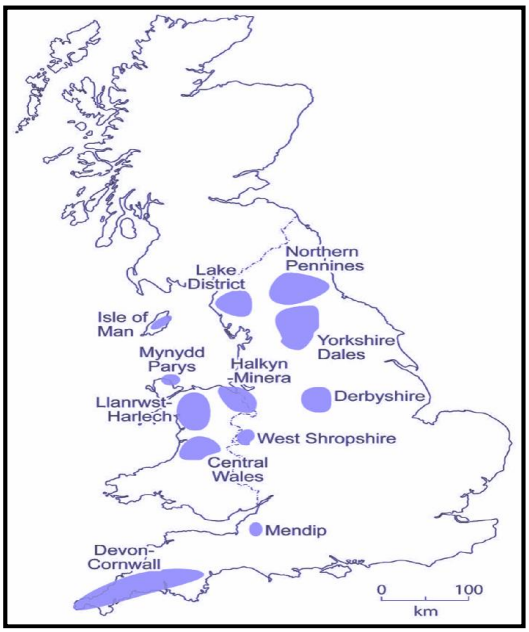

Figure 6.9: Metal mining areas of England and Wales ((Dunham et al., 1978)

The last major mine closed in the late twentieth century and currently there are no operating metal mines in England and Wales. Early mining methods were primitive. Separation of metal sulphide ore minerals were carried out by crushing and gravity sorting in nearby streams, and in many areas solid and liquid mining effluents were disposed off directly into the nearest river. In some areas, especially in North Pennines and Yorkshire Dales, a hydraulic form of mining known as “hushing” was carried out by constructing dams upslope of mineral veins, then opening sluices to release water that eroded overburdened and exposed metal ores for easy excavation. The resulting torrent exposed underlying bedrock and mineral veins, which were worked using simple quarrying methods. All these methods involved water and led to the transfer of significant quantities of metal rich sediments to the river

systems. The table below lists the river catchments most affected by mining related metal contamination (Table 6.2).

Water Framework Directive River Basin District	Catchment	Ore field
Dee	Clywedog	Halkyn-Minera
Humber	Swale, Wharfe, Nidd, Ure	Yorkshire Dales
	Ecclesbourne, Hamps, Manifold, Derwent	Southern Pennines (Derbyshire)
Northumbria	South Tyne, Wear, Tees	Northern Pennines
North West	Newland's Beck, Coledale Beck	Lake District
Severn	Rea Brook	West Shropshire
	upper Severn	Central Wales
	Yeo, Axe	Mendip
Solway Tweed	Glenridding Beck	Lake District
South West	Camel, Erme, Fal, Fowey, Gannel, Tamar	Devon-Cornwall
Western Wales	Afon Goch Twymyn, Rheidol, Ystwyth	Mynydd Parys Central Wales

Table 6.2: River basins most affected by metal mining in England and Wales (Hudson-Edwards et al., 2008)

In England and Wales, discharge of metal-enriched mine drainage water is common, not only from metal mines, but also from coalmines, which is a very important factor in the North-East of England. Studies on uptake of cadmium, lead and zinc concentrations by plants and crops growing on metal contaminated flood plains in England and Wales have shown that the plants and crops contain high concentration of these metals which exceeded concentrations in plants from uncontaminated areas (Macklin and Lewin, 1989).

In spite of large number of mining related contaminated river systems in the world, the exact nature of its effect on human population living beside, and relying on, these rivers for food and drinking water is relatively unknown. Several studies have measured the concentration of metals in river systems

affected by metal mining in England and Wales (Macklin and Dowsett, 1990, Macklin and Lewin, 1989). Some of the most intensely studied catchments are the rivers flowing to North Sea, which drain the Northern Pennines and Yorkshire Dales. River waters in these catchments exhibit high dissolved cadmium, lead and zinc concentration. This is mainly due to contamination from old mining areas upstream, although industrial activity, sewage treatment and atmospheric fallout also contribute (Table 6.3). This is highly relevant to the present study since individuals in these areas are within the catchment area of the Freeman Hospital for hepato-biliary and pancreatic diseases.

The Environment Agency proposes a scheme to assess contaminated sediments and soils in metal mining affected rivers in England and Wales. The scheme involves:

1. Identify catchments potentially impacted by sediment/water-borne metal contamination.
2. Assess the extent and magnitude of contamination
3. Carry out risk assessments in areas identified above to fully investigate source-pathway-receptor relationship
4. Develop and implement contaminant monitoring and remediation programmes if risks are unacceptable.

Catchment	Lead Dissolved (µg l^{-1})	Zinc Dissolved (µg l^{-1})	Cadmium Dissolved (µg l^{-1})	Reference
Wear	**9.31**	**1770**[5]	**0.32**[9]	Environment Agency
West Allen	**52**	**1562**[5]		Gozzard *et al.*, 2006
West Allen	**8.1**	**736**[5]	**2.17**[9]	Environment Agency
Swale (upper)	**175**	-	**0.8**[10]	Environment Agency
Ure	0.89	14.3		Neal *et al.*, 1997
Nidd	**16.7**	19.4[5]	**0.25**[8]	Environment Agency
Ouse	2.25	17.9		Neal *et al.*, 1997
Ouse	**11.7**	9.14		Environment Agency
Wharfe	1.51	16.7		Neal *et al.*, 1997
Aire	1.29	31.0		Neal *et al.*, 1997
Derwent	0.28	14.2		Neal *et al.*, 1997
Calder	1.25	35.9		Neal *et al.*, 1997
Don	0.84	26.9		Neal *et al.*, 1997
Trent	1.21	29.3		Neal *et al.*, 1997
Tamar	0.03 - 3.28	3.4 - 12.7	0.03 - 0.07	Rawlins *et al.*, 2003
Tamar	4.9	11.6	**0.181**[8]	Environment Agency
Ystwyth		**170 - 880**[3]		Grimshaw *et al.*, 1976
Ystwyth	**92**	**363**[3]		Fuge *et al.*, 1991
Ystwyth	**121**	**3040**[3]	**5.5**[7]	Environment Agency
Rheidol	**9.5**	**285**[3]		Fuge *et al.*, 1991
Rheidol	**12.4**	**145**[3]	**0.34**[7]	Environment Agency
Conwy	2.7 - **16.3**	**94 - 3260**[5]		Elderfield *et al.*, 1979
Conwy	**17 - 50**	**300 - 342**[5]		Brydie & Polya, 2003
Conwy	**27.7**	**2485**[3]	**9.8**[10]	Environment Agency
Environmental quality standards	7.2[1]	8 - 125[2]	0.08 - 0.25[5]	

All samples were passed through a 0.45 µm filter. Neal *et al.* (1997) values are averages of weekly samples from 1993-1995.
[1]Pb annual average (dissolved) EQS set for the Water Framework Directive.
[2]Zn annual average (total) suitable for salmonid (game) fish. EC Dangerous Substances Directive (76/464/EEC). Hardness (as $CaCO_3$) related standard.
[3]Zn EQS = 8 µg l^{-1} (total Zn, hardness related)
[4]Zn EQS = 50 µg l^{-1} (total Zn, hardness related)
[5]Zn EQS = 75 µg l^{-1} (total Zn, hardness related)
[6]Cd annual average (dissolved) EQS set for WFD. Hardness (as $CaCO_3$) related.
[7]Cd EQS = 0.08 µg l^{-1} (dissolved Cd, hardness related)
[8]Cd EQS = 0.09 µg l^{-1} (dissolved Cd, hardness related)
[9]Cd EQS = 0.15 µg l^{-1} (dissolved Cd, hardness related)
[10]Cd EQS = 0.25 µg l^{-1} (dissolved Cd, hardness related)

Table 6.3: Dissolved metal concentration in water samples from some mining impacted English and Welsh rivers. Values in bold exceed the environment quality standard (EQS) (Hudson-Edwards et al., 2008)

The Environmental Agency is already carrying out identification of catchments at risk from sediment-borne metal contamination. The WFD River Basin Characterisation maps, and investigation on non-coal mines should be used to identify catchments potentially impacted by contamination (Figure 6.10) (Jarvis et al., 2008, Hudson-Edwards et al., 2008).

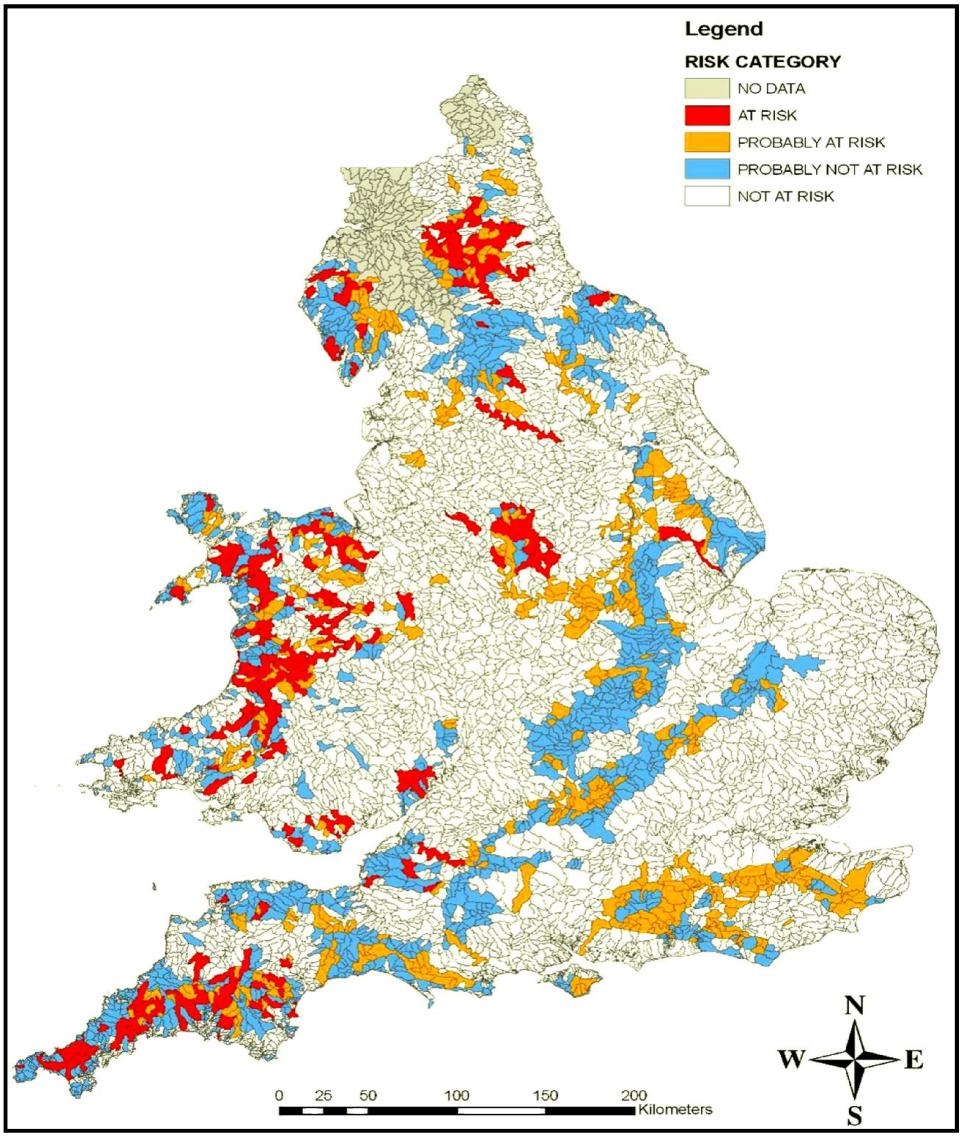

Figure 6.10: Map showing catchments of England and Wales with likely ('at risk and 'probably at risk') to have sediment-borne, mining related metal contamination (after Environment Agency, 2008)

Once the potentially impacted catchments are identified, the next step will be to assess the extent and magnitude of contamination. This would involve a detailed look onto the mining history of the area and the geomorphology of the catchments. The Environment Agency's Indicative Floodplain Maps can be used to delineate river corridors and extent of contamination. Two examples are given below on the use of these flood maps (Figure 6.11, Figure 6.12).

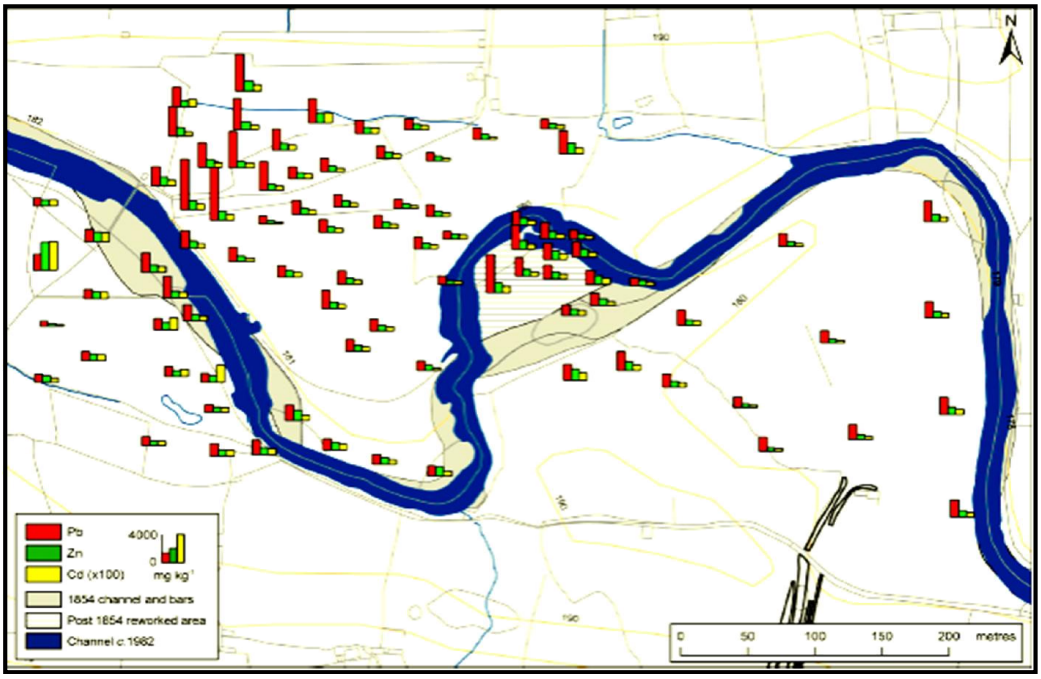

Figure 6.11: Geomorphology and sediment-borne metal concentrations along River Swale, North-East England (Brewer et al., 2005)

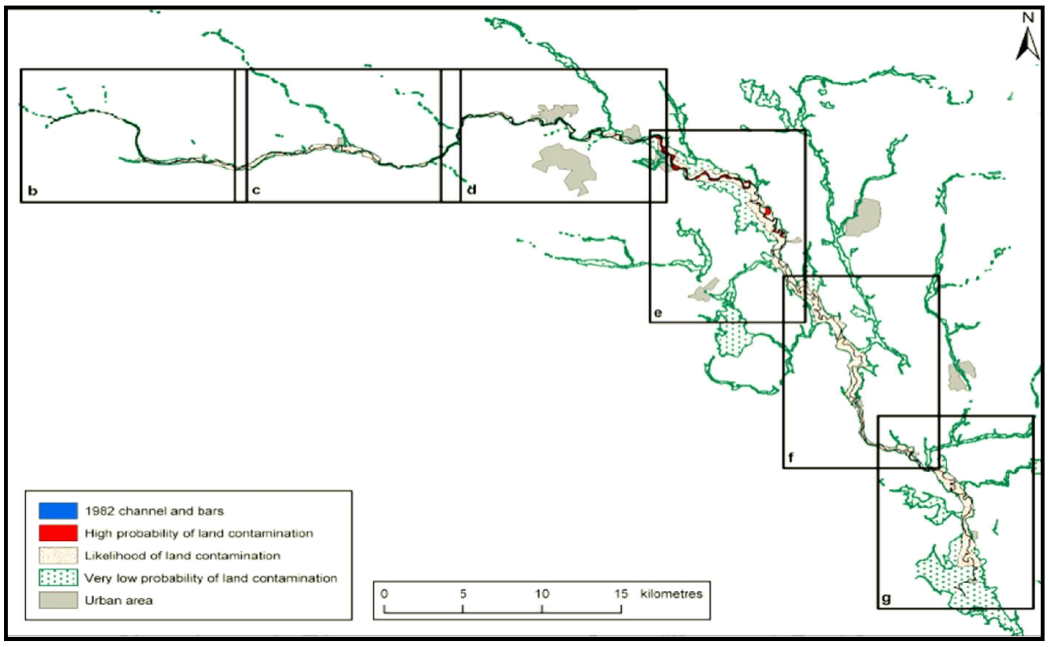

Figure 6.12: Example of floodplain map showing probability of contamination along River Swale (Brewer et al., 2005)

The above discussion and the report by Environment Agency have highlighted the significantly elevated concentrations of metals such as cadmium, lead and zinc in river sediments and floodplain soils and in river

water as a result of now abandoned metal-mining activity. The Environment Agency recommended close monitoring of the effect of this contamination and its impact on the environment and health. Our project to look at the possible association of metal exposure and development of pancreatobiliary cancer will make a definite contribution to understand the impact on health due to environmental pollution, which is an extremely important issue in both developed and developing countries.

Chapter 7

Conclusion and the Future of the Project

7. Conclusion and the Future of the Project

It has been emphasised in the discussion that environmental pollution with metals has gradually gained considerable importance. The association between different heavy metal contamination of the environment and cancer has been investigated for a long time but no direct cause and effect relationship has yet been established. Heavy metals such as lead, cadmium, mercury, arsenic etc have been extensively studied and their effects on human health regularly reviewed by international bodies such as the WHO, including possible contribution to the aetiology of different types of cancers.

The emphasis of this project was on a very specific type of cancer, i.e. pancreatobiliary malignancies. As demonstrated in our results section, this group of cancers is by no means homogeneous and comprises pure pancreatic cancers, pure biliary cancers (gallbladder cancers or cholangiocarcinoma) and periampullary cancers, where the cell of origin can be from pancreatic, biliary or intestinal epithelium. There is considerable evidence to show that clinically and prognostically, each subset behaves in a different way. It is very interesting to note, however, that anatomically these lesions are very close together and one would expect that the cells are exposed to similar environmental effects. In other words, any possible environmental influence on the aetiology of these tumours is likely to influence all the different subsets of the disease.

In recent years, 2 important developments occurred which increased our interest in heavy metals and pancreatobiliary cancers. One was the evidence

generated by organizations like the Environment Agency that there is definite evidence of heavy metal contamination of the environment in this country. The second development was the emerging evidence of the association of heavy metals and malignancy from different parts of the world. Evidence from Egypt, India and United States regarding the association of cadmium and lead with pancreatic cancer were the first suggestions of an association between heavy metals and pancreatobiliary malignancy. This is also supported by the high cadmium content in tobacco, which is a definite risk factor for pancreatic cancers.

Compared to the above-mentioned countries, the degree of environmental pollution is much lower in the UK. But even from the North-East of England there are studies demonstrating an association between environmental pollution and lung cancer. The association between heavy metal pollution and pancreatobiliary malignancy has not been looked into in details in this country.

We looked at the biliary concentration of the heavy metals in our study instead of serum or urinary concentration, which has been done in other similar projects. Our rational was that the metal, which is excreted in bile, is in direct contact with the cells that have eventually turned malignant and it seemed logical to look at the immediate environment of these cells to consider the influence of these metals on cell growth.

It is very important to note that the presence of cadmium or lead in significantly higher concentration in the malignant group of patients does not mean a cause and effect relationship between the concentration of these metals and the development of cancer. It suggests the association between the two and adds to the evidence that this association really exists. It also opens up the possibility of further research to confirm this association and to establish a cause and affect relationship if it exists.

There are 2 major projects that can emerge out of the present one. One would be to carry out an experimental study in a controlled environment and expose commercially available biliary and pancreatic cell lines to different heavy metals to demonstrate possible genotoxic or mitogenic effects. The other project would be a detailed epidemiological study to look at all cancer patients from a particular catchment area, e.g the Tees River catchments and record their possible lifetime environmental exposure to different metals and correlate with the pollution data available from the Environment Agency.

Chapter 8

Summary of work in Public Domain

8. Summary of Work in Public Domain

1. Role of heavy metals in pancreato-biliary malignancy. **Sen G**, Mansfield SD, Gallagher PV, Green P, Rimmer D, Manas DM, Charnley RM. *Oral presentation at the 6th World Congress of IHPBA at Washington DC, June 2-6, 2004*
2. Role of heavy metals in pancreato-biliary malignancy. **Sen G**, Mansfield SD, Gallagher PV, Green P, Rimmer D, Manas DM, Charnley RM. *Presented at 6th Congress of the European Hepato-Pancreato-Biliary Association, Heidelberg, Germany, May 25 – 28, 2005*
3. Biliary heavy metal excretion and its association with pancreato-biliary malignancy. **G Sen**, S D Mansfield, P Green, D Rimmer, D M Manas, R M Charnley. *Presented at the 8th Congress of the European Hepato-Pancreato-Biliary Association, Athens, Greece, June 18 – 20, 2009*
4. Biliary heavy metal excretion and its association with pancreato-biliary malignancy. **G Sen**, S D Mansfield, P Green, D Rimmer, D M Manas, R M Charnley. *Oral presentation at the 4th Alpine HPB Meeting, Italy, February 2009.* ***Winner of Covidien Travel Bursary****.*

9. Appendix I (Consent Form)

Name:

D.O.B.

Unit no:

A study to investigate the causes of biliary tract cancers

CONSENT FORM FOR OBTAINING BILE

This is a consent form that you are asked to sign in order to take part in a research project.

A doctor should have discussed the study with you and given you an information sheet before you sign this consent form.

You should not feel pressurised to take part in this study; if you choose not to be included in the study, your care and treatment will not be affected.

I agree to take part in the study of the causes of biliary tract cancers. I understand that bile will be taken during the normal course of my investigation and treatment. The bile will be stored and analysed later. As the results from the analyses will only be used for research purposes I understand that participants are not told of their individual results.

I understand that the research may or may not be of direct benefit to me.

I have received and read an information sheet about this study.

Signature: ..... Date:.....

Name: .....

To be completed by the person obtaining consent.

I have explained the study and witnessed the signature.

Signature: ..... Date:.....

Name: .....

10. Appendix II (Information Sheet for patients)

A STUDY TO INVESTIGATE THE CAUSES OF BILIARY TRACT CANCERS

INFORMATION FOR PATIENTS

You have a problem with the biliary tract or gallbladder that requires investigation or treatment. We would be grateful if you could help in a research project where bile from the biliary system is obtained from you. We are interested in why cancers of the bile ducts develop.

The biliary tract consists of tubes, the proper name is ducts, which drain the liver into the bowel. These ducts are present both inside and outside the liver. Cancers of the biliary tract are uncommon and it is uncertain why they develop. Many possible factors have been described as potential.

Research from India has suggested that one of these factors may be heavy metals, such as lead or cadmium. It is not known if this could be an important factor in people who live in Britain. We would like to obtain a sample of your bile to test for the amount of heavy metals. The bile is obtained without any extra difficulty during the normal course of your investigations and treatment.

Many diseases run in families, with some members of the family but not others developing the disease. It is unknown if biliary tract cancers run in the family or if there may be problems with the genes that may interact with the environment. New genetic problems are constantly being identified. We would like a sample of your blood to store, in case any tests in the future prove to be useful. The blood sample will only be used for genetic analysis in relation to biliary tract cancers.

As the results from the analyses will only be used for research purposes participants are not told of their individual results. All information gathered will be treated as strictly confidential. No individual will be identified in any medical research literature resulting from this study.

You do not have to participate in this study. If you choose not to be included in the study your care and treatment will not be affected. You are also quite free to change your mind and withdraw from the study at any time.

If at any time you have any questions that you would like to ask regarding this study you should address them to Mr. R. M. Charnley, Consultant Surgeon (ext 26147) or Mr. Gourab Sen, Clinical Fellow (Bleep 3393), the Department of Surgery, Freeman Hospital.

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